Identificación de biomarcadores de cáncer de laringe en pacientes candidatos a terapias de preservación de órgano.

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Identification of novel laryngeal cancer biomarkers in patients treated with organ preservation approaches.

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Trabajo realizado en el Hospital Universitario Virgen del Rocío en colaboración con el Instituto de Biomedicina de Sevilla (IBIS).

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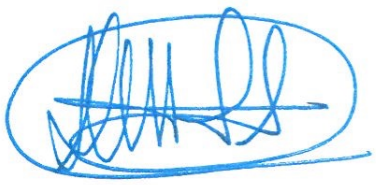
CERTIFICAN:

Que han revisado el trabajo de investigación titulado "Identification of novel laryngeal cancer biomarkers in patients treated with organ preservation approaches – Identificación de biomarcadores de cáncer de laringe en pacientes candidatos a terapias de preservación de órgano" realizado por María José de Miguel Luken con DNI 74868886-E, y ratifican como Director y tutor del mismo la autorización para su presentación, al reunir la condiciones requeridas para ser leído y defendido ante el tribunal correspondiente como Tesis para optar al grado de doctor con mención internacional.

Doctorando: Dña. María José de Miguel Luken



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A mi madre, de quien he aprendido a levantarme tras cada caída,
y me ha acompañado en cada etapa del Camino

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Si vuelvo la vista atrás a todos esos años que pasé en Sevilla, me vienen muchísimos recuerdos, casi todos ellos buenos. En aquel tiempo aprendí a ser lo que siempre quise ser, oncóloga médica. Y en ese camino me encontré con muchas personas que fueron cambiando mi vida. Entre esos nombres a los que no sabría muy bien en qué orden definir mejor está Manuel Chaves Conde. Tan amigo como buen profesional y mentor, me ha enseñado cómo ser buen médico, luchar por el bienestar de los pacientes, luchar contra las adversidades laborales, mantener la cabeza fría y seguir adelante con integridad. Mané fue quien, con la idea de que la investigación debe beneficiar en su orden final al paciente, me ofreció buscar biomarcadores en cáncer de laringe que pudieran cambiar en algún momento la práctica clínica, su propia consulta de cáncer de cabeza y cuello.

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Parte investigadora, parte docente, pero sobre todo hermana y amiga, Verónica de Miguel ha sido una de las piezas clave para la culminación de esta tesis. Desde cómo hacer ecuaciones en el colegio, pasando por las integrales del instituto, la bioestadística en la facultad y hasta la actualidad, es un ejemplo de tesón y rigurosidad que me ha guiado y ayudado desde la infancia.

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levantarme una y otra vez, igual que cuando éramos pequeños. Caminante no hay camino, se hace camino al andar. Y nosotras hemos andado mucho juntas.

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SUMMARY

Laryngeal cancer represents approximately 1% of the total of malignancies with an estimated incidence age-standardised rate of 4.4 per 100.000 persons. It is most commonly presented in males at their sixth decade of life. Anatomically, the larynx is an air passage and an organ of phonation that extends from the tongue to the trachea. Two different histologies are described in the larynx: the pseudostratified respiratory epithelium that covers the inner aspects of the larynx, and the non-keratinized, stratified squamous epithelium that covers the vocal folds and the exterior surfaces of the larynx. The vast majority of tumours arising the larynx are conventional squamous cell carcinomas (SCC), although there are other less frequent subtypes. SCC of the head and neck is considered now to be the final stage of a multi-step process in which loss of heterozygosity, amplifications, deletions, up and down-regulation oncogenes or tumour-suppressor genes take part. The primary cause for head and neck cancer is tobacco use, where even a low quantity of cigarette smoking has been associated with an increased risk. Alcohol consumption is the second major risk factor, which potentiates the effects of tobacco. The most extended cancer staging system is the tumour node metastasis (TNM) system by the American Joint Committee on Cancer (AJCC). For non-metastatic patients, treatment options depend on whether the tumour is presented at early stages, or as locoregional disease. In general, early stages are treated with either surgery or definitive radiotherapy, while advanced stages require a multimodal approach. Three sparing approaches are accepted: radiotherapy, bio or chemotherapy with concomitant radiotherapy and induction chemotherapy followed by radiotherapy with or without bio/chemotherapy. Although functional organ sparing approaches allow larynx preservation, they do not provide a survival advantage over surgery. Moreover, those treatments confer significant toxicities and approximately 30-40% of patients will relapse or lead to an incompetent larynx. Predictive biomarkers would facilitate pre-treatment identification of patients who are unlikely going to be cured by radiation-based therapy. Managing these patients with surgery rather than with preservation approaches, local disease control and survival could be potentially optimized and unnecessary treatment related morbidities from unsuccessful larynx treatments avoided. With this study we aim to find biomarkers that could be related with prognosis and being used as predictive biomarkers for pre-treatment patient selection. We evaluated 65 patients with larynx cancer treated at the Hospital Universitario Virgen del Rocío from August 2005 until February 2014. Eligibility criteria for treatment preservation include patients with stage II-IV laryngeal tumours that had no contraindication for chemotherapy and/or radiotherapy, significant cartilage destruction, or more than 2 cm tumoral invasion of the base of the tongue. Patients were mainly male with stage III supraglottic SCC and with a good general condition. Histological characterization of all samples was done by hematoxylin and eosin

staining, followed by immunohistochemistry analysis of tissue microarrays. Analysed markers included proliferation markers such as Ki67 or the activated form of ERK, apoptosis such as mutant p53 or activated AKT, and finally markers involved in the DNA-damage response (DDR) pathway such as MAP17, SGLT and pH2AX. Subsequent statistical analysis was performed, taking into consideration clinical and pathological factors. Concerning clinical factors, T4 primary tumour extension and patients who needed a pre-treatment tracheotomy had worse laryngoesophageal dysfunction-free survival (LDS) and seemed not to benefit of preservation treatments. Moreover, receiving an optimal platinum dose determined LDS, as patients unable to complete treatment had worse prognosis. In what respects to pathological factors, markers related with the DDR were associated with survival. DDR is a network of cellular pathways that sense, signal and repair DNA lesions. MAP17 is a small membrane protein highly expressed in metastatic carcinoma. Its expression is associated with a SGLT-dependent reactive oxygen species (ROS) increase. While a mild increase in ROS activates signalling cascades that upregulate tumorigenic processes, further ROS increases lead to toxic cellular environment and to programmed cell death. Moreover, γH2AX is a component of the histone octamer in nucleosomes involved in recruiting DNA repair proteins in response to the presence of DNA double-strand breaks. Hence, it´s increasing is related to structural DNA damage. Therefore, DNA-damage produced by ROS would lead to pH2AX increasing. Laryngeal cancer tumours with high levels of ROS producing MAP17 and SGLT proteins and with high pH2AX expression should benefit from therapies such as cisplatin or radiotherapy that increase oxidative stress and could sensitize them to cell death. Our analysis confirmed a significant relationship between high MAP17 protein expression and increased overall survival (OS), locoregional control (LRC) and LDS. In fact, high MAP17 levels demonstrated better OS than low levels (67 months vs. 31.7 months, IC 95%; $p<0.001$). In addition, high-MAP17 and high-SGLT showed improved OS, better than MAP17 alone. In our cohort, pH2AX was related to LDS (high-pH2AX HR 0.26, $p = 0.02$). When analysed together pH2AX expression and dose of cisplatin received during radical treatment, there was a significant correlation with survival and LDS. Also, patients with high-MAP17 and high-pH2AX showed to have better OS and LDS. Therefore, this work suggests that high levels of MAP17 induced ROS that in turn increases DNA-damage and DDR signalling, measured as high-pH2AX. Upon further DNA-damage and further increase in ROS molecules induced by cisplatin and RT treatment, tumours with higher oxidative stress, are more suitable to undergo apoptosis. The inherent DDR pathway activation biomarkers MAP17 and pH2AX are valuable prognostic markers in patients with laryngeal carcinoma who received organ preservation approaches.

RESUMEN

El cáncer de laringe representa aproximadamente el 1% del total de las neoplasias, con una incidencia estimada estandarizada por edad de 4.4 cada 100.000 habitantes. Es más frecuente en varones durante la sexta década de vida. Anatómicamente, la laringe se extiende desde la lengua hasta la tráquea y conduce el aire siendo el órgano responsable de la fonación. Se compone fundamentalmente de dos tipos histológicos: epitelio respiratorio pseudoestratificado en la cara interna, y el epitelio no-queratinizado que envuelve las cuerdas vocales y la cara externa. La gran mayoría de los tumores laríngeos son carcinomas epidermoides, aunque existen otros subtipos menos frecuentes. Los cánceres de cabeza y cuello (CCyC) se consideran el estadio final de un complejo proceso que incluye pérdidas de heterocigosidad, amplificaciones, deleciones y alteraciones de la regulación de oncogenes y genes supresores de tumores. La causa más fundamental del CCyC es el tabaco, donde hasta un bajo consumo del mismo se ha asociado a un aumento del riesgo de cáncer. El segundo factor es el alcohol, que potencia el efecto del tabaco. El sistema de clasificación más frecuentemente utilizado es el TNM por sus siglas en inglés "tumour, node, metastasis" elaborado por la American Joint Committee on Cancer (AJCC). El tratamiento de los pacientes no metastásicos depende de si la enfermedad se ha presentado en un estadio inicial o como enfermedad locoregional. En general, los estadios iniciales se benefician de cirugía o radioterapia, mientras que los avanzados requieren de un abordaje multidisciplinar. Tres estrategias terapéuticas están aceptadas: radioterapia, bio o quimioterapia concomitante con radioterapia y quimioterapia de inducción seguida por radioterapia con o sin bio/quimioterapia. Aunque las terapias de preservación de órgano permiten conservar la laringe, no han demostrado aumentar la supervivencia en comparación con cirugía. Además, estos tratamientos conllevan importantes toxicidades y en un 30-40% los pacientes sufren recidivas o pierden la funcionalidad de la laringe. Disponer de biomarcadores predictivos facilitaría la identificación de aquellos pacientes que no se van a beneficiar de tratamiento basado en la radioterapia. Derivando estos pacientes directamente a cirugía, el control local de la enfermedad y potencialmente la supervivencia podrían optimizarse y evitar toxicidades innecesarias. Con este estudio se pretende identificar biomarcadores pronósticos que puedan llegar a ser predictivos para la selección de pacientes. Se han evaluaron 65 pacientes con cáncer de laringe tratados en el Hospital Universitario Virgen de Rocío entre agosto de 2005 y febrero de 2014. Los criterios de selección para la preservación de órgano incluyeron estadios II a IV de cáncer de laringe, sin contraindicación para quimio o radioterapia, ni destrucción significativa de cartílago o invasión mayor de 2 cm de la base de la lengua. Los pacientes fueron mayoritariamente varones con carcinomas epidermoides estadio III y buen estado general. Se procedió a la caracterización histológica de todas las muestras mediante tinción de hematoxilina-eosina seguido de

inmunohistoquímica de los microarrays. Se han estudiado marcadores de proliferación como Ki67 y la forma activada de ERK, de apoptosis como la mutación de p53 o la forma activada de AKT y finalmente marcadores implicados en los mecanismos de reparación del daño de ADN (RDA) como MAP17, SGLT y pH2AX. Posteriormente se llevó a cabo el estudio estadístico tomando en consideración tanto los factores clínicos como histopatológicos. Respecto a los factores clínicos, la extensión del tumor primario T4 y aquellos pacientes que precisaron de traqueotomía previa al tratamiento obtuvieron una peor supervivencia con laringe funcionante (SLF) y parecen no beneficiarse de tratamientos de preservación de órgano. Por otro lado, recibir una dosis óptima de platino durante el tratamiento influyó en la SLF ya que aquellos que no lo completaron tuvieron un peor pronóstico. Respecto a los factores moleculares, las marcadores relacionados con la RDA se asociaron a supervivencia. Los mecanismos RDA conforman una red de diagnóstico, señalización y reparación de las lesiones producidas en el ADN. MAP17 es una proteína de membrana altamente expresada en carcinomas. Dicha expresión se asocia a un aumento de las especies de oxígeno reactivo (EOR) dependiente de SGLT. Mientras que incrementos moderados de EOR activan cascadas de señalización que activan los procesos tumorigénicos, un aumento más significativo conlleva a un ambiente celular tóxico dando lugar a la muerte celular programada. Por otro lado, γH2AX es un componente del octámero de la histona del nucleosoma encargado de reclutar proteínas de reparación de ADN en respuesta al daño de la doble cadena de ADN. Por tanto, su aumento se relaciona con daño de ADN. Ya que ROS produce daño de ADN, pH2AX debería aumentar en este contexto. La hipótesis es que aquellos tumores laríngeos con niveles altos de ROS producidos por MAP17 y SGLT, y con expresión de pH2AX podrían beneficiarse de terapias como el cisplatino y la radioterapia, que aumentan el estrés oxidativo y dar lugar a la muerte celular. El análisis confirmó que existe una relación entre MAP17 y el aumento de la supervivencia global (SG), control locoregional (CLR) y SLF. Niveles altos de MAP17 se asociaron a un aumento de SG estadísticamente significativo comparado con niveles bajos (67 meses vs. 31.7 meses, IC 95%; $p<0.001$). La combinación de niveles altos tanto de MAP17 como de SGLT obtuvieron mejor SG que MAP17 solo. Por otro lado, pH2AX se correlacionó con SLF (alto-pH2AX HR 0.26, $p = 0.02$). El análisis conjunto de pH2AX junto con una dosis óptima de platino también se correlacionó con SG y SLF, demostrando resultados similares el análisis de pH2AX junto con alto-MAP17. Por tanto, niveles altos de MAP17 inducen EOR dando lugar a un aumento del daño de ADN y de los procesos de RDA, medido indirectamente por el aumento de pH2AX. En este tipo de tumores con alto estrés oxidativo, el sucesivo daño producido por terapias basadas en platino y la radiación tienen una mayor sensibilidad a la apoptosis. Por tanto, los biomarcadores implicados en los mecanismos de RDA MAP17 y pH2AX son marcadores pronósticos para aquellos pacientes con cáncer de laringe candidatos a terapias de preservación de órgano.

1.1 LARYNX CANCER EPIDEMIOLOGY

Around 157.000 new cases of laryngeal cancer were diagnosed worldwide in 2012, which represents approximately 1% of the total of malignancies. The estimated general incidence age-standardised rate (ASR) is 4.4 per 100.000 persons, with a broad difference between males and females (8.8 versus –vs- 0.8). In Europe, laryngeal cancer is the 20th most common tumour, with 39.900 new cases diagnosed in 2012; in contrast, the United States (US) registered 12.300 new cases in the same period. Worldwide, the highest incidence rate is found in the Caribbean area and are lowest in Western Africa, but this may just reflect varying data quality worldwide though [(1)].

On the other hand, estimated general mortality ASR is 2.1 per 100.000 persons; 4.3 males and 0.3 women. Worldwide, the highest ASR mortality rates for laryngeal cancer are in Hungary for men and Albania for women [(1)]. In contrast, the lowest rates are found in Iceland for both men and women. Mortality differs significantly between European countries. Males have lowest mortality rates in Northern Europe (ASR 1.9) followed by Southern Europe (ASR 4.3) and finally Eastern Europe (ASR 7.0) (Figure 1). Spain has similar mortality rates to the rest of Southern Europe countries, with a male ASR of 4.3 and female ASR of 0.3; however, those numbers are still far away from Northern countries [(2)] (Table 1).

Larynx cancer in Spain	**Incidence**			**Mortality**			**5-year prevalence**		
	Total	(%)	ASR	Total	(%)	ASR	Total	(%)	ASR
All population	3.182	1,5	4,1	1.321	1,3	1,5	11.200	1,9	28,3
Male	2.914	2,3	7,8	1.235	1,9	2,9	10.246	3,1	52,7
Female	268	0,3	0,7	86	0,2	0,2	954	0,4	4,7

ASR: age-standarized rate.

***Table 1**. Estimated Incidence, Mortality and 5-year prevalence in Spain, 2012. Source: Spanish Society of Medical Oncology annual report [(6)].*

The incidence of laryngeal cancer is falling in developed countries, because of the success of risk factors prevention campaigns. However, population growth and increased aging still results still in a larger number of total annual cases. During the last twenty years, larynx cancer has decreased globally by a 25% [(3)]; for example, in the US the rate of decreasing is about 2% to 3% per year [(4)] (Figure 2). In Spain, the incidence in 2012 was 3182 cases, and the prediction of the World Health Organization (WHO) for 2020 is of 3735 new cases. However, this slight increase in total numbers translates a decreasing rate of 19.4% since 2003-07 [(5)]. Therefore, the incidence of laryngeal carcinoma shows a decreasing trend for for both sexes in our country similar to the rest

of Europe and the US. But also within Spain, there is a significant variability. In the period 2003-2007, male incidence varied from 8.9 per 100.000 habitants in Albacete to 13.1 in the Basque Country. For females, Cuenca was found to be the region with a lower incidence and the Basque Country the highest rate (0.1 vs 0.8/100.000). Therefore, the Basque Country is the autonomous community with a higher incidence of laryngeal cancer. This variability is influenced by the differences in the prevalence of alcohol and tobacco consumption between regions [5].

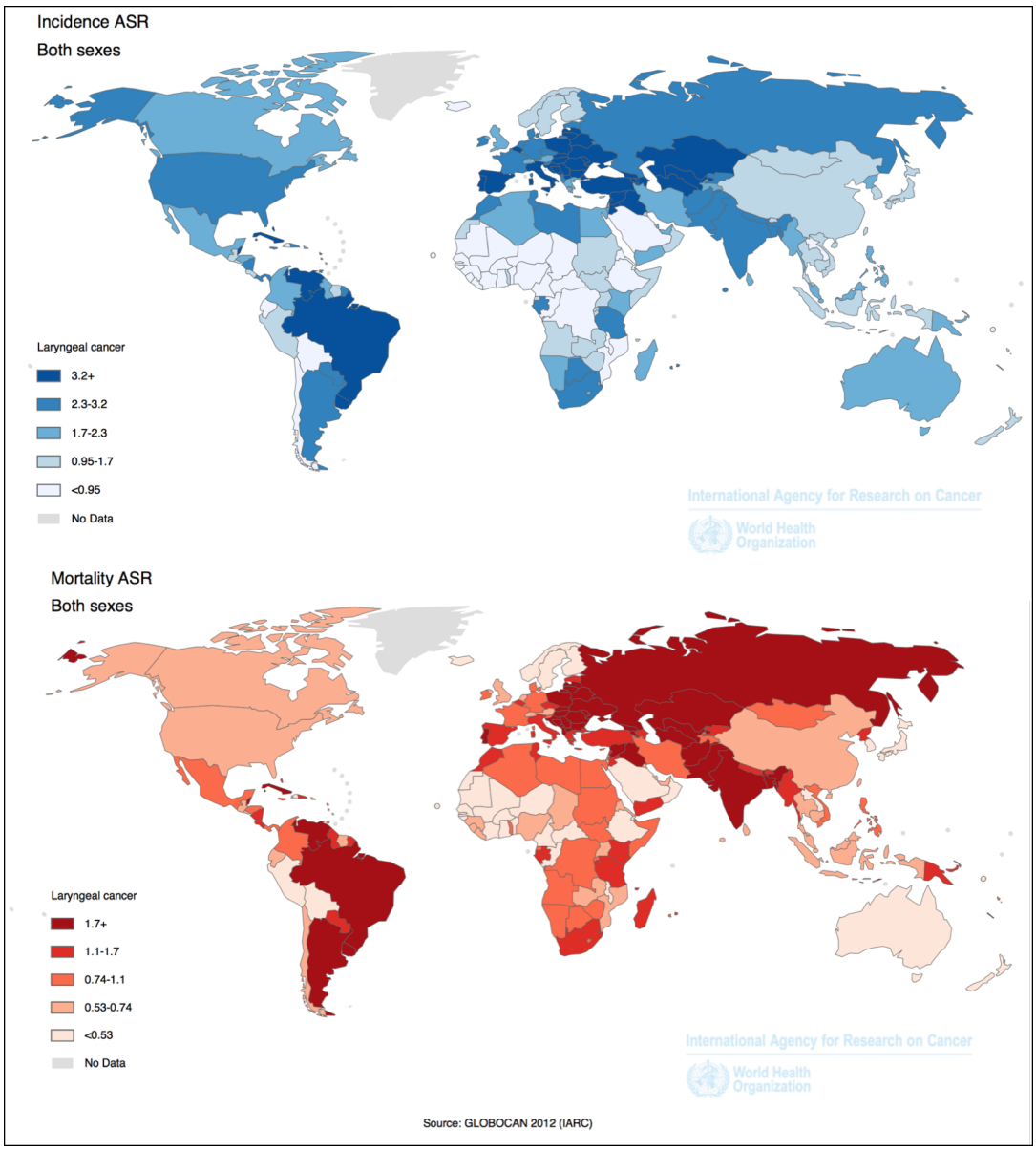
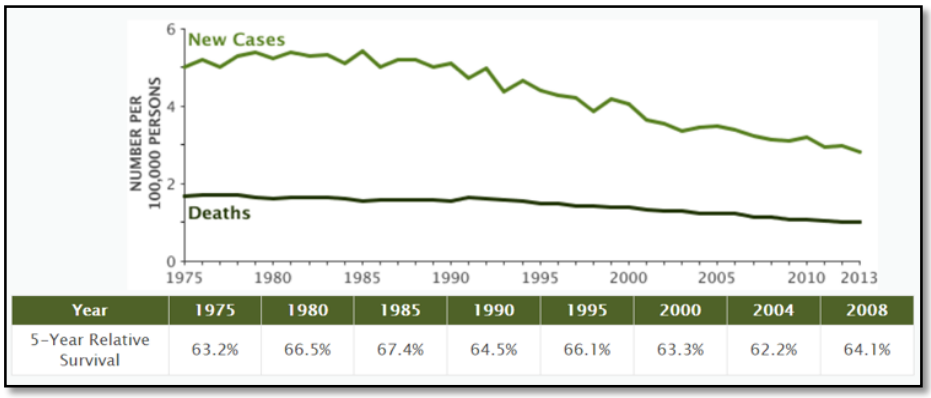


***Figure 1**. Worldwide laryngeal cancer incidence and mortality. Source: International Agency for research on cancer - World Health Organization. GLOBOCAN 2012.*

However, the Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute in the US, has established that 5-year relative survival has slightly dropped during the last decades. In numbers, the 5-year survival decreased by a 3.3% from 1985 until 2008. Although this fact has coincided in time with the implantation of preservation approaches, they were not broadly used and standardized by then and major conclusions may not be extrapolated [(4)]. Interestingly, in a recent publication it was found that in the US, uninsured patients or Medicaid patients (joint federal-state program that provides health coverage) had a decreased relative survival when compared to privately insured individuals [(7)], being one of the facts that could modify general survival. On the other hand, in the United Kingdom (UK) statistics show a trend towards improved survival over the last decades. A 5.5% increasing of the 5-year relative survival has been observed from 1990 till 2011 (69.8% survival in the period 2010-2011) [(8)]. In Spain, 5-year relative survival was around 60% survival in the period of 2000-2007, so in the range of developed countries [(6)].



Year	1975	1980	1985	1990	1995	2000	2004	2008
5-Year Relative Survival	63.2%	66.5%	67.4%	64.5%	66.1%	63.3%	62.2%	64.1%

Figure 2. *Number of new cases, deaths and 5-year relative survival in US. Source: SEER Cancer Statistics.*

Median age at presentation is 65 years, where it is most frequently diagnosed among people aged 55-64, in a 30.9% of the cases [(4)]. This is similar for both developed and emerging countries, as some series in India showed maximum incidence between the 60-69 years (31.93%) [(9)]. In the US, at the time of diagnosis most patients have locoregional disease (77%); of them, 55% localized disease and 22% regional disease. As metastatic disease is found as a later event in laryngeal cancer, only 19% were found to have them [(4)]. In conclusion, laryngeal cancer is more prevalent in males than females, aged around the sixth decade of life, and frequently diagnosed as localized or locoregional disease. There are significant incidence variations worldwide that depends mainly on risk factors habits. In Europe, laryngeal cancer rates are dropping but there are still differences between North, Central and South regions. Finally, Spain behaves similar to other Mediterranean countries, but there are areas where the incidence is still high.

1.2 ANATOMY AND HISTOLOGY OF THE LARYNX. PATTERNS OF SPREAD. TNM STAGING

1.2.1. General Anatomy, histology and pathology

General Anatomy

The larynx is an air passage and an organ of phonation that extends from the tongue to the trachea. It projects ventrally between the great vessels of the neck and is covered anteriorly by skin, fasciae and the infrahyoid strap muscles that lower the hyoid bone and the larynx. Above, it opens into the laryngopharynx and forms its anterior wall; below, it continues into the trachea. Until puberty, male and female larynges are similar in size. After puberty, the male larynx enlarges considerably in comparison with the female.

Skeletal framework. The skeletal framework of the larynx is formed by cartilages interconnected by ligaments and fibrous membranes. The laryngeal cartilages are the single thyroid, cricoid and epiglottic cartilages, and the paired arytenoid, cuneiform and corniculate cartilages (Figure 3). The hyoid bone is not part of the larynx but provides the muscular attachments from above that aid in laryngeal motion. Larynx cartilages are joined to surrounding structures by extrinsic membranes. It is also interconnected by intrinsic ligaments and fibrobroelastic membranes, of which the thyrohyoid and quadrangular membranes, together with the conus elasticus, are the most significant. The thyrohyoid membrane is external to the larynx, whereas the paired quadrangular membranes and conus elasticus are internal. The named ligaments are the anterior cricothyroid ligament, the hyoepiglottic and thyroepiglottic ligaments, and the cricotracheal ligament.

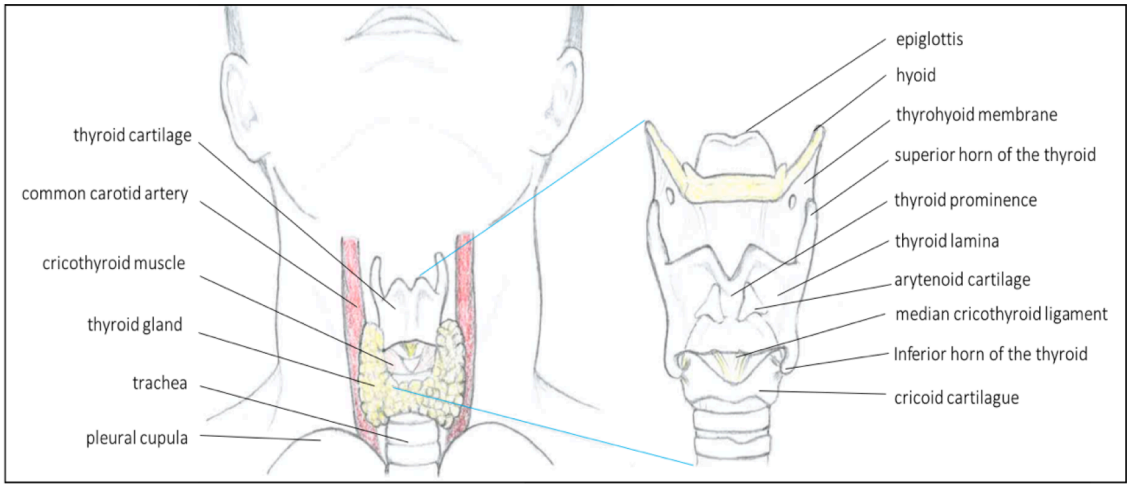


Figure 3. *General laryngeal anatomy. Version adapted from the Human Anatomy Atlas, Netter 2nd Edition.*

Laryngeal cavity. The walls of the cavity are formed of fibroelastic membranes and lined with mucous membrane that folds over the free edges of these membranes within the larynx. On either side, the continuity of the fibroelastic membrane is interrupted between the upper vestibular and lower true vocal folds. The folds project into the lumen of the cavity and divide it into upper and lower parts, separated by a middle portion between the two sets of folds that leads into the laryngeal ventricle. The upper folds are the vestibular (ventricular or false vocal) folds; the median aperture between them is the rima vestibuli. The lower pair is the (true) vocal folds, primary source of phonation.

In tumor staging the supraglottis refers to all those parts of the larynx that lie above the glottis, meanwhile the glottis is defined as the anterior and inferior surfaces of the true vocal folds and the anterior and posterior commissures. Finally, the subglottis is the region below the glottis that extends to the inferior border of the cricoid cartilage.

Muscles. The muscles of the larynx may be divided into extrinsic and intrinsic groups. The extrinsic muscles connect the larynx to neighbouring structures and are responsible for moving it vertically during phonation and swallowing, thus opposite to the infrahyoid muscles that lower the larynx. They include the infrahyoid strap muscles, thyrohyoid, sternothyroid and sternohyoid, and the inferior constrictor muscle of the pharynx. The extrinsic muscles can affect the pitch and the quality of the voice by raising or lowering the larynx, and geniohyoid elevates and anteriorly displaces the larynx, particularly during deglutition.

The intrinsic laryngeal muscles may be placed in three groups according to their main actions. The posterior and lateral cricoarytenoids and oblique and transverse arytenoids vary the degree of abduction and adduction of the vocal folds and thus the dimensions and the degree of opening of the rima glottidis. The cricothyroids, posterior cricoarytenoids, thyroarytenoids and vocalis regulate the length and tension of the vocal folds. The third group of muscles is the oblique arytenoids, aryepiglottic and thyroepiglottic muscles, which modify the laryngeal inlet.

Vascular supply and inervation. The blood supply of the larynx is derived mainly from the superior and inferior laryngeal arteries. Rich anastomoses exist between the corresponding contralateral laryngeal arteries and between the ipsilateral laryngeal arteries. The superior laryngeal arteries supply the greater part of the tissues of the larynx, from the epiglottis down to the level of the vocal cords, including the majority of the laryngeal musculature. The inferior laryngeal artery supplies the region around cricothyroid, while its posterior laryngeal branch supplies the tissue around posterior cricoarytenoid. The larynx is innervated by the internal and external branches of the superior laryngeal nerve, the recurrent laryngeal nerve and sympathetic nerves. Conventionally, the internal laryngeal nerve is described as sensory, the external laryngeal

nerve as motor, and the recurrent laryngeal nerve as mixed.

Lymphatic drainage. The upper deep cervical lymph nodes act as pathways for the spread of malignant tumours of the supraglottic larynx. Up to 40% of these tumours will have undergone such spread at the time of clinical presentation, mainly along the superior laryngeal artery draining to the deep cervical lymph nodes at the bifurcation of the common carotid artery. On the contrary, the glottis is very poorly endowed with lymphatic vessels; some 95% of malignant tumours confined to the glottis will present with no spread to adjacent lymph nodes. Tumours of the subglottic larynx will often spread to the paratracheal lymph node chain prior to clinical presentation. The paratracheal lymph nodes occupy a deep-seated position in the root of the neck (Figure 4) and so their enlargement may remain occult [10-16].

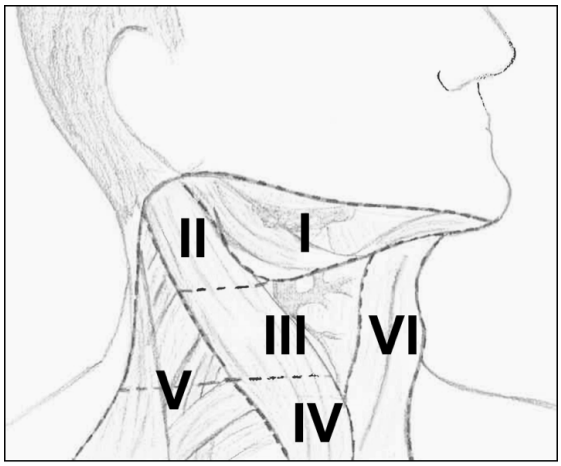


Figure 4. *Cervical lymph node chains.*

Laryngeal histology

The laryngeal mucosa is continuous with that of the pharynx above and the trachea below. Over the vocal folds, it is thinner and is firmly attached to the underlying vocal ligaments. The laryngeal epithelium is mainly a ciliated, pseudostratified respiratory epithelium that covers the inner aspects of the larynx, and it provides a mucociliary clearance mechanism shared with most of the respiratory tract. The vocal folds, however, are covered by non-keratinized, stratified squamous epithelium; this important variation protects the tissue from the effects of the considerable mechanical stresses that act on the surfaces of the vocal folds. The exterior surfaces of the larynx, which merge with the laryngopharynx and oropharynx, are subject to the abrasive effects of swallowed food, and are therefore also covered by non-keratinized, stratified squamous epithelium (Figure 5). The laryngeal mucosa has numerous mucous glands, especially over the epiglottis, and along the margins of the aryepiglottic folds anterior to the arytenoid cartilages. [11, 17].

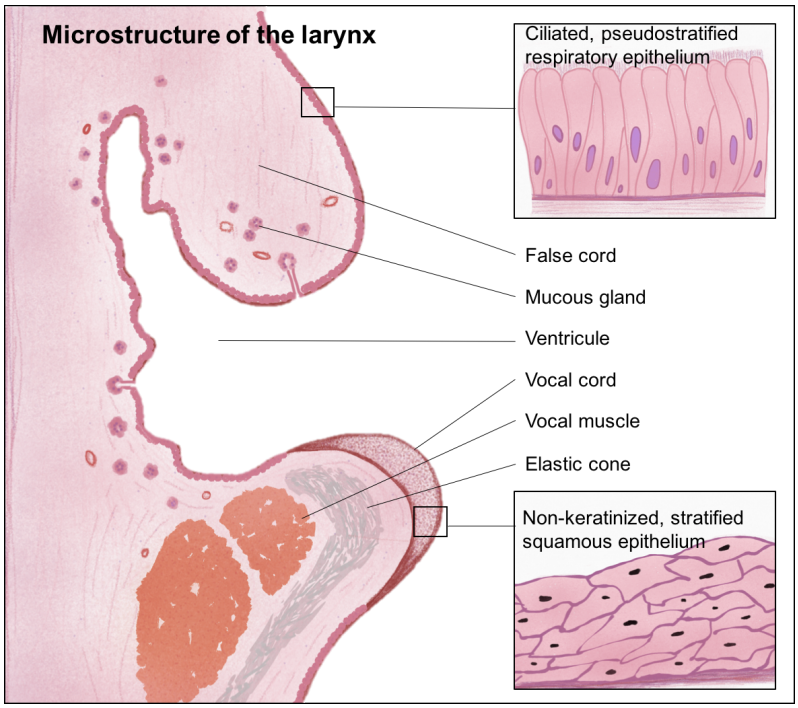


Figure 5. *Laryngeal cavity histology subtypes.*

Laryngeal pathology

The vast majority of all laryngeal malignancies (95%) are conventional squamous cell carcinomas (SCC). They vary according to their degree of differentiation to well, moderate and poor carcinomas. Glottic cancers are generally well differentiated and have a less aggressive behaviour in comparison with carcinomas at the other sites of the larynx. SCC often arises in a background of mucosal squamous dysplasia or carcinoma in situ and typically presents islands, tongues and clusters of atypical cells invading the laryngeal stroma. Features of squamous differentiation also comprise individual cell keratinisation, intercellular bridges and keratin pearls [(17)].

Recognition of the less common variants is significant because their biological behaviour is often different from conventional SCC.

Verrucous SCC: comprises approximately 1% to 4% of laryngeal malignancies. It presents as a locally invasive fungating mass that can be confused with a benign process and characteristically does not metastasize to regional lymph nodes. Histologically, it appears broad pegs of highly differentiated squamous cells invading the laryngeal stroma in a pushing pattern, and mitoses rarely found. Surgical excision is the cornerstone of therapy since radiotherapy is associated to poor results and with the possibility of anaplastic transformation of the tumour [(18)].

Spindle cell carcinomas (SpCC) are a rare variant of the squamous cell carcinoma (SCC). It is more predominant in males with a male-to-female ratio of nearly 7:1. Smoking, alcohol consumption, and previous irradiation are predisposing factors. It presents usually as a polypoid or less as an endophytic mass. SpCC consist of elongated (spindle) cells of epithelial origin, representing an unusual form of poorly differentiated SCC though morphologically these resemble a sarcoma. The most common site of origin in the head and neck region is the glottis and hypopharynx. In comparison with SCC, laryngeal SpCC appear to be more likely to present at an earlier stage. The metastatic potential of both entities seems to be similar. Surgery is considered to be the mainstay in the management of SpCC, as the effectiveness of radiotherapy was not confirmed in recent studies and the role of chemotherapy remains unclear [19].

Basaloid SCC: usually presented at an advanced stage with neck metastases. Microscopically, the tumour is characterized by nests and lobules of small basaloid cells with a high nuclear-to-cytoplasmic ratio and areas demonstrating abrupt squamous differentiation, foci of carcinoma in situ or invasive SCC. Comedonecrosis and stromal hyalinosis are also common findings. Surgery of the primary tumour and neck and radiation therapy is the usual therapeutic approach [20].

Other tumors such as minor salivary gland tumors are rare; even rarer are soft tissue sarcomas, lymphomas, small cell neuroendocrine carcinoma, and plamacytomas. Hemangiomas, chondromas, and osteochondromas are reported, but their malignant counterparts are rare [21].

1.2.2. Patterns of spread

According to the location of the primary tumour there are different patterns of spread:

Supraglottis

Suprahyoid Epiglottis. Lesions may grow producing exophytic mass with little tendency to destroy cartilage or spread to adjacent structures. Others may infiltrate and destroy cartilage. The tend to invade the vallecula, pre-epiglottic space, lateral pharyngeal walls, and the rest of the supraglottis.

Infrahyoid Epiglottis. Lesions tend to produce irregular tumour nodules with invasion through the porous epiglottic cartilage into the preepiglottic space. They grow circumferentially to involve the false cords, aryepiglottis folds, and eventually, the medial wall of the pyriform sinus and the pharyngoepiglottic fold [21, 22].

Lymphatic drainage is initially to the level II nodes and then to levels III and IV. The incidence of clinically positive nodes is 55% at diagnosis, 16% are bilateral [23].

Glottis

Early false cord carcinomas usually have the appearance of a submucosal mass and are difficult to delineate accurately. They extend toward the thyroid cartilage and medial wall of the pyriform sinus. Extension to the infrahyoid epiglottis is common. Initial invasion of the vocal cord may occur submucosally and may be difficult to detect. Vocal cord invasion is often associated with thyroid cartilage invasion.

On the other hand, the majority of lesions of the vocal cord begin on the free margin and upper surface and are easily visible. When diagnosed, about two-thirds are confined to one cord, usually the anterior two thirds of the cord. Extension to the anterior commissure is frequent. As the lesion enlarges, it extends to the ventricle, false cord, vocal process of the arytenoids, and subglottis. Infiltrative lesions invade the vocal ligament and thyroarytenoid muscles, eventually reaching the thyroid cartilage. The conus elasticus initially acts as a barrier to subglottic extension. Advanced glottis lesions eventually invade through the thyroid cartilage or thyrocricoid membrane to enter the neck and/or thyroid gland [21, 22].

The incidence of clinically positive nodes at diagnosis varies with tumour stage: T1, 1% or less, T2, 5% or less; and T3 or T4, 20-30% [24]. Supraglottic spread is associated with metastasis to the level II nodes. Anterior commissure and subglottic invasion is associated with levels III and IV and Delphian node involvement [25].

Aryepiglottic fold and arytenoid

Early lesions are usually exophytic. As the lesions advance, they extend to adjacent sites and eventually cause laryngeal fixation due to invasion the the cricoarytenoid muscle and joint. Advanced lesions invade the base of the tongue, pharyngeal wall, and postcricoid pharynx.

Subglottic larynx

They are in general uncommon. It is difficult to determine whether a tumour started on the under surface of the vocal cord or in the subglottis with extension to the cord. They involve the cricoid cartilage early, because there is no intervening muscle layer. Cord fixation is common [21]. A 10% incidence of clinically positive lymph nodes has been reported. Spread is primarily to the Delphian nodes and the level IV nodes [26].

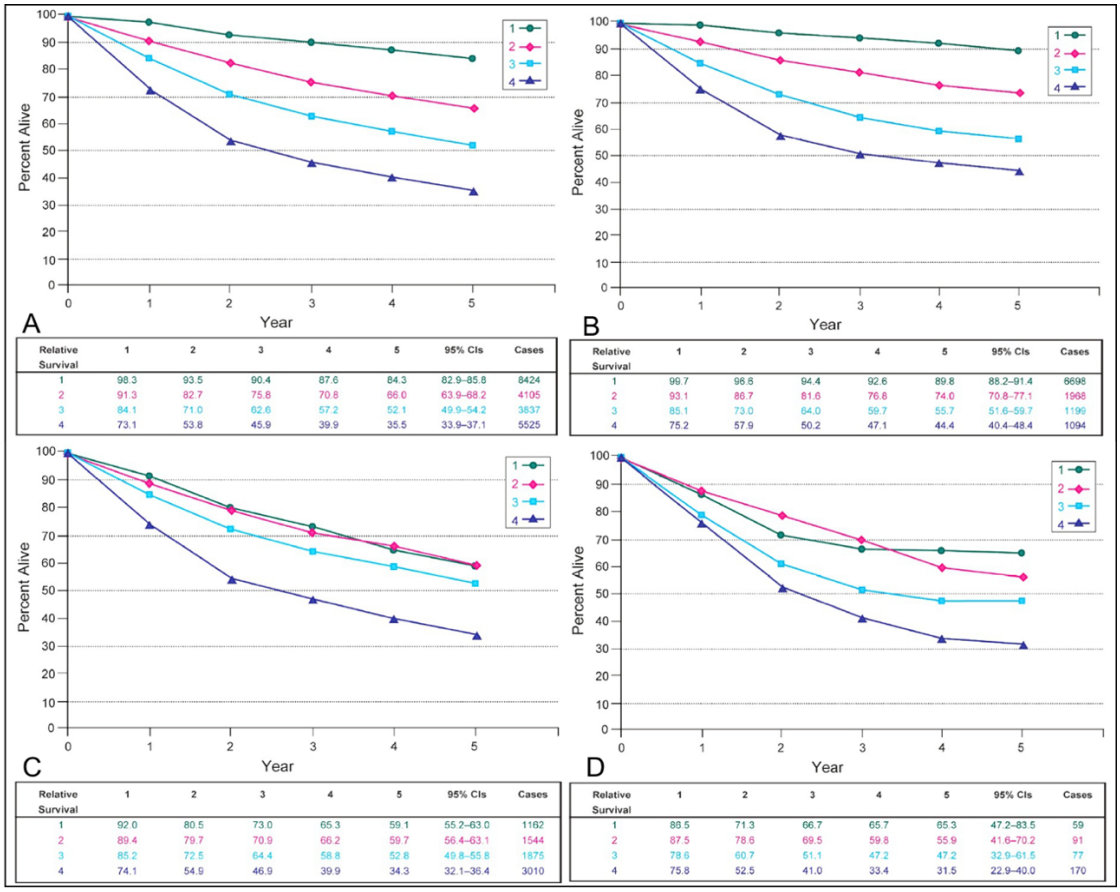
1.2.3. AJCC staging and survival

The extend of cancer at the time of diagnosis is a key factor that defines prognosis and is a critical element in determining appropriate treatments based on the experience and outcomes of groups of prior patients with similar stage.

Several cancer staging systems are used worldwide. The most clinically useful staging system is the tumour node metastasis (TNM) system maintained collaboratively by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC). The TNM system classifies cancers by the size and extent of the primary tumour (T), involvement of regional lymph node (N), and the presence or absence of distant metastases (M). The last edition of the TNM classification is the 7th published in 2010 which has been the one used for this work.

Site	**Subsite**
Supraglottis	Suprahyoid epiglottis
	Infrahyoid epiglottis
	Aryepiglottic folds, arytenoids
	Ventricular bands (false cords)
Glottis	True vocal cords, including anterior and posterior commissures
Subglottis	Subglottis

Table 2. *Anatomical laryngeal subsites. Source: AJCC, TNM classification, 7th edition.*



A	Relative Survival	1	2	3	4	5	95% CIs	Cases
	1	98.3	93.5	90.4	87.6	84.3	82.9–85.8	8424
	2	91.3	82.7	75.8	70.8	66.0	63.9–68.2	4105
	3	84.1	71.0	62.6	57.2	52.1	49.9–54.2	3837
	4	73.1	53.8	45.9	39.9	35.5	33.9–37.1	5525

B	Relative Survival	1	2	3	4	5	95% CIs	Cases
	1	99.7	96.8	94.4	92.6	89.8	88.2–91.4	6698
	2	93.1	86.7	81.6	76.8	74.0	70.8–77.1	1968
	3	85.1	73.0	64.0	59.7	55.7	51.6–59.7	1199
	4	75.2	57.9	50.2	47.1	44.4	40.4–48.4	1094

C	Relative Survival	1	2	3	4	5	95% CIs	Cases
	1	92.0	80.5	73.0	65.3	59.1	55.2–63.0	1162
	2	89.4	79.7	70.9	66.2	59.7	56.4–63.1	1544
	3	85.2	72.5	64.4	58.8	52.8	49.8–55.8	1875
	4	74.1	54.9	46.9	39.9	34.3	32.1–36.4	3010

D	Relative Survival	1	2	3	4	5	95% CIs	Cases
	1	88.5	71.3	66.7	65.7	65.3	47.2–83.5	59
	2	87.5	78.6	69.5	59.8	55.9	41.6–70.2	91
	3	78.6	60.7	51.1	47.2	47.2	32.9–61.5	77
	4	75.8	52.5	41.0	33.4	31.5	22.9–40.0	170

Figure 6. *Five-year relative survivals by combined AJCC stage, 1998-1999. A: SCC of the larynx. B. carcinoma of the glottis. C. carcinoma of the supraglottis. D. Carcinoma of the subglottis. Source: AJCC, TNM classification, 7th edition.*

Although the regional lymph node and metastasis classification is common for the different sites of the larynx, the primary tumour is divided into three parts: supraglottis, glottis and subglottis. Also within these three parts there are different subsites as it can be seen in Table 2 [(27)].

Survival depends on the location of the primary tumour, where the glottis has a better survival (90% survival for stage I), followed by subglottis (65% survival for stage I). This difference is maintained for all T stages [(27,28)] (Table 3).

Definitions of TNM

Primary tumor (T)	
Supraglottis	
T1	Tumor limited to one subsite of supraglottis with normal vocal cord mobility.
T2	Tumor invades mucosa on more than one adjacent subsite of supraglottis or glottis or region outside the supraglottis without fixation of the larynx.
T3	Tumor limited to larynx with vocal cord fixation and/or invades any of the following: postcricoid area, preepiglottic space, paraglottic space , and/or inner cortex of thyroid cartilage.
T4a	Moderately advanced local disease. Tumor invades through the thyroid cartilage and/or invades tissues beyond the larynx.
T4b	Very advanced local disease. Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures.
Glottis	
T1	Tumor limited to the vocal cord(s) (may involve anterior or posterior commissure) with normal mobility.
T1a	Tumor limited to one vocal cord.
T1b	Tumor involves both vocal cords.
T2	Tumor extends to supraglottis and/or subglottis and/or with impaired vocal cord mobility.
T3	Tumor limited to the larynx with vocal cord fixation and/or invasion of paraglottic space, and/or inner cortex of the thyroid cartilage.
T4a	Moderately advanced local disease. Tumor invades through the outer cortex of the thyroid cartilage and/or invades tissues beyond the larynx.
T4b	Very advanced local disease. Tumor invades prevertebral space, encases carotid artery, or mediastinal structures.
Subglottis	
T1	Tumor limited to the subglottis.
T2	Tumor extends to vocal cord(s) with normal or impaired mobility.
T3	Tumor limited to larynx with vocal cord fixation.
T4a	Moderately advanced local disease. Tumor invades cricoid or thyroid cartilage and/or invades tissues beyond the larynx.
T4b	Very advanced local disease. Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures.

Regional Lymph nodes	
Nx	Regional Lymph nodes cannot be assessed.
N0	No regional lymph node metastasis.
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension.
N2	Metastasis in single or multiple ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension, or in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension.
N2a	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension.
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimentsion.
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension..
N3	Metastasis in a lymph node, more than 6 cm in greatest dimension.

Distant Metastasis	
M0	No distant metastasis
M1	Distant metastasis

Anatomic Stage/Prognosis Group			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3 T1 T2 T3	N0 N1 N1 N1	M0 M0 M0 M0
Stage IVA	T4a T4a T1 T2 T3 T4a	N0 N1 N2 N2 N2 N2	M0 M0 M0 M0 M0 M0
Stage IVB	T4b Any T	Any N N3	M0 M0
Stage IVC	Any T	Any N	M1

Table 3. AJCC laryngeal cancer staging. 7th Edition.

1.3 RISK AND PROGNOSTIC FACTORS FOR LARYNGEAL CANCER

1.3.1. Risk factors of laryngeal cancer

The primary cause for head and neck cancer (HNC) is tobacco use [29]. There is a 7-fold increased risk of developing laryngeal cancer for smoker patients and, even though the risk drops for former smokers, it is still more than 4-folf compared to never smokers [30]. Also, a low quantity of

cigarette smoking is associated with an increased risk of head and neck cancer. The results from the INHANCE consortium pooled analysis showed that smoking 3-5 cigarettes per day was particularly predictor of laryngeal cancer with an odds ratio (OR) of 3.48. However, the risk for low smoking frequency was not found among smokers with smoking duration shorter than 20 years, suggesting that low frequency of cigarette consumption contributes to HNC, but that smoking duration seems to play at least an equal or stronger role [31].

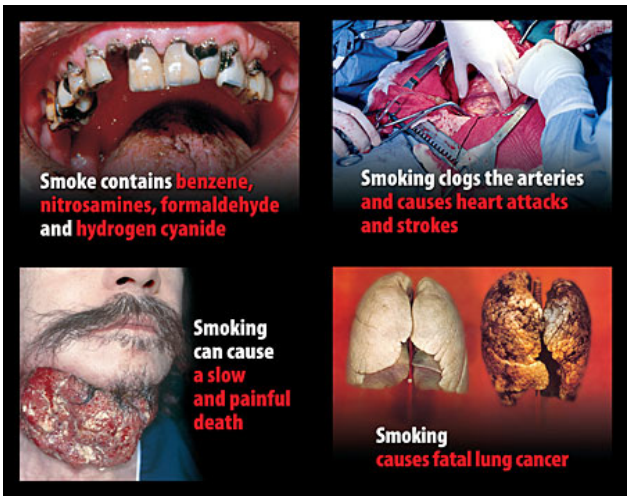


Figure 7. *European Commission campaign for tobacco prevention approved in 2005.*

Source: http://www.who.int/tobacco/healthwarningsdatabase/

In the same way, it has been observed that young patients had a higher proportion of oral tongue cancer and oral cavity/oropharynx cancer with a lower proportion of larynx cancer than older adult cases, most probably because of the reduced length of exposure to tobacco [32]. Cigar or pipe smoking elevate also HNC risk (OR 3.49 and 3.71, respectively) [33]. In addition, survival duration, risk of recurrence, vulnerability to a second primary neoplasm, and treatment efficacy are also affected by continued smoking after HNC diagnosis and treatment. Survival is influenced by whether the patient ceases to smoke once diagnosed or continues to smoke during of following treatment [34-35]. There has been found a relationship between continued smoking and survival among 115 patients with stage III or IV squamous cell carcinoma of the head and neck. Thirty-nine percent of patients who smoked lived at least 2 years after diagnosis, compared with 66% of non-smokers [35]. Decreased survival in patients that continue smoking is associated to the risk of recurrence but also to second primary tumours development. In a study, patients who continued to smoke were twice as likely to experience a disease recurrence compared with patients who abstained following treatment [36]. Current smokers are four times as likely to develop a second primary tumour compared to former smokers, and this risk increased with the duration and

intensity of smoking. Particularly in laryngeal cancer, it has been seen that risk of a second primary tumour was proportional to the level of exposure [37, 38]. Data also suggest that smoking may have an adverse effect on the response to medical treatment. In a prospective trial with more than one hundred patients it was seen that patients that stopped smoking during trial had better results than those who did not (75% of complete tumour responses vs 45%) [35]. Also in terms of toxicities and comorbidities, patients that continue smoking have more treatment-related complications. Mucositis for example takes a longer time to heal in smoker patients than in non-smokers during radiotherapy treatment [39]. Moreover, population with HNC has also an increased risk of deep venous thrombosis, pulmonary embolism, and impaired wound healing following surgery which compromise treatments and outcomes [40, 41]. Taking into account the risk of cancer development, cardiovascular disease and other related diseases to tobacco, it is estimated that smoking kills nearly 6 million people each year worldwide, more than HIV/AIDS, tuberculosis and malaria combined. Tobacco consumption imposes a considerable cost upon society, including direct healthcare costs and indirect costs due to absenteeism and premature death. In the European Union for the year 2000, cost was estimated to be approximately €363 billion [42].

Alcohol is the second major risk factor for laryngeal cancer, which potentiates the effects of tobacco. The association between alcohol drinking and laryngeal cancer was first reported in a case-control study in the 1950s [43]. In 1987, the International Agency for Research on Cancer working group classified alcoholic beverages as ‘‘carcinogenic to humans” and concluded that laryngeal cancers are causally related to the consumption of alcoholic beverages [44], a conclusion that was reaffirmed in a more recent evaluation in 2009 [45]. The risk is proportional to the amount of ethanol drunk per day, as a meta-analysis reported in 2001. Laryngeal cancer relative risk (RR) adjusted by smoking status was 1.29 (1.23–1.36), 1.68 (1.53–1.84), and 2.72 (2.36–3.30) for drinking 25, 50, and 100 g of ethanol per day, respectively [46]. However, in another meta-analysis, significant association was not found for light alcohol drinking (≤1 drink/day), RR = 0.88; 95% CI: 0.71–1.08), whereas moderate drinking (>1 to <4 drinks/day) was associated with a 1.5-fold increase in risk (RR = 1.47; 95% CI: 1.25–1.72) and heavy drinking (≥4 drinks/day) with a 2.5-fold increased risk (RR = 2.62; 95% CI: 2.13–3.23). Overall, alcohol drinking versus non-drinking was associated with an approximately 2-fold increase in risk of laryngeal cancer. In this meta-analysis, subgroup analyses for studies that adjusted for main potential confounding factors (age, sex, and tobacco use) and several further subgroup analyses showed similar results, which suggest the robustness of the results [47].

Human papillomavirus (HPV) is emerging as an important HNC factor in developed countries, especially in oropharyngeal tumours. HPV-induced HNC has distinct epidemiology and biology, and a more favourable prognosis compared to HPV negative tumours. In patients with oropharyngeal carcinoma treated with chemoradiotherapy, HPV-positive had better 3-year

overall survival rates than patients with HPV-negative tumours (82.4%, vs. 57.1%; P<0.001) [48]. In another trial with more than five thousand patients with HNC it was observed that the hazard ratio for death in patients HPV positive was 0.42 and that they had a better response to therapy. Positivity for HVP was found in 22% of patients, most of them having the HPV 16 positive genotype [49,50]. However, it is known that there is a low rate of HPV-positive tumours among the laryngeal cancer population, which is estimated in around 1.6-6.5%. Therefore, although HPV is emerging as an important risk factor that correlates with prognoses in HNC, the role in laryngeal cancer it is probably less relevant and it is not yet stablished [51-53].

A recent meta-analysis supports the association of exposure to asbestos with an increased risk of laryngeal cancer mortality among male workers, with a standardized mortality rate of 1.69 [54]. It has been also suggested that there is an additive joint effect between asbestos exposure and alcohol consumption, and a more than additive joint effect between asbestos exposure and tobacco consumption, as well as between the three of them together [55]. In a multicentre case-control study conducted in four European countries it was evaluated the role of occupational exposures and risk of laryngeal and hypopharyngeal cancer. Elevated risks for ever exposure to coal dust were found for both hypopharyngeal (OR= 4.19, 95% CI: 1.18, 14.89) and laryngeal cancer (OR= 1.81, 95% CI: 0.94, 3.47), with clear dose-response patterns. Laryngeal cancer was significantly associated with exposure to hard-alloys dust (OR = 2.23, 95% CI: 1.08, 4.57) and chlorinated solvents (OR = 2.18, 95% CI: 1.03, 4.61), without dose-response relations. A possible link between high formaldehyde exposure and laryngeal cancer was also suggested but further studies are required [56]. Gastroesophageal reflux is another risk factor for laryngeal cancer as it was shown is a systematic review where the pooled odds ratio (OR) was 2.21 (95%, CI= 1.53-3.19), regardless of age, gender, tobacco and alcohol consumption [57].

Genetic susceptibility has been implicated in HNC. Emerging phenotypic and genotypic data support the idea of genetic susceptibility for HNC. In an historical cohort study the RR for HNC was 7.89 (CI-1.50 to 41.6) in first degree relatives of patients with multiple primary head and neck cancer [58]. In a more recent pooled analysis with 8.967 HNC cases and 13.627 controls, having a family history of HNC in first-degree relative increased the risk of HNC 1.7, which was higher when the affected relative was a sibling (OR= 2.2, 95% CI 1.6-3.1) rather than a parent (OR=1.5, CI 1.1-1.8) and for more distal HNC anatomic sites (hypopharynx and larynx) [59].

Instead, vegetable, fruits, and vitamin C intake might reduce the risk of laryngeal cancer. Higher intakes of vitamin C were inversely related to laryngeal cancers (OR = 0.52, 95% CI: 0.40-0.68) in a pooled analysis in the International Head and Neck Cancer Epidemiology Consortium [60]. In another study, total vegetable and fruit consumption was inversely associated with risk of HNC

(OR=0.61, CI 0.44-0.85) and all HNC subtypes, with the strongest associations for oropharyngeal cancer [61].

1.3.2. Clinical and pathological prognostic factors

For laryngeal cancer, the main prognostic factor for overall survival (OS) is tumour staging. Pathologic tumour volume was found to be an independent predictive factor for distant metastasis, overall survival, disease-free survival and locoregional recurrence [62]. T4 primary extension and more than 2 cm tumoral invasion of the base of the tongue increased salvage laryngectomy in the Veterans study [63]. Moreover, lymph node invasion and extracapsular extension are also independent predictive factors for survival [62]. Location of the primary tumour also plays a significant role in prognosis, as supra and subglottic cancers have worse prognosis than glottis cancers, probably in relation to earlier detection, and later node extension [27,28]. Other prognostic factors are patient's comorbidity and performance status-ECOG (PS) [64], most probably because frail patients are not suitable candidates for aggressive treatment management. Finally, surgical resection margins affection and pretreatment need for a tracheotomy have been related to poor disease-free survival [65,66]. However, none of those factors have been validated for treatment decision-making so far.

1.4 MOLECULAR BIOLOGY OF LARYNGEAL CANCER

1.4.1. Genetic susceptibility

Genetic polymorphisms variants in tobacco carcinogen and alcohol metabolism genes may increase HNC risk. GSTM1 null genotype appears to confer increased HNC and particularly laryngeal increased risk (OR=1.22, 95% CI 1.1-1.36) [67, 68]. The variant Val allele of the CYP1A1 Ile462Val polymorphism is another consistent susceptibility maker for HNC, with a 35% increased risk in a meta-analysis of 12 studies [68]. Presenting fast metabolizing alleles for alcohol dehydrogenase (ADH), ADH1B and ALDH2 genes, resulted in increased acetyladehyde levels and associated with HNC significantly interacting with alcohol consumption [69]. In a case-control study single nucleotide polymorphisms (SNPs) in nucleotide excision repair (NER) genes such as ERCC5, ERCC6 and RAD23B could modify laryngeal cancer risk. In particular, ERCC6 showed a decreased risk, while ERCC5 and RAD23B increased it [70]. Furthermore, XPD and ERCC1 may be associated to poor disease free survival (DFS) in HNC, suggesting a significant role of NER in these tumours [71].

1.4.2. Cytogenetic alterations

Malignant progression may be associated to particular chromosomal alterations. For instance, early changes at 3p, 4q, 8p, 9p, 11q, 13q and 17p have been observed in leukoplakias while loss of heterozygosity (LOH) of 9p21 is a common genetic event in oral premalignancies [72,73]. Califano et at described a model of carcinogenesis in 1996, but it seems to be more the accumulation rather than the order of genetic events what determines tumoral progression (Figure 8) [74].

Amplifications/loss of heterozygosity

Amplification in a variety of key genes have been described in HNC, such as of 3q, 3q24-qter, 5p, 8q23-24, 11q13, 11q14-22, 18p, 18q11.2, and 19q among others [75, 76]. The critical proto-oncogene Cyclin D1 is amplificated within the 11q13 region and may be a marker of progression in primary HNC [77]. Cyclin D1 overexpression has been reported in laryngeal carcinoma and might be implicated in regulating cell proliferation by the critical G1/S checkpoint [78]. P63 is a p53 homologue and a potential oncogene in squamous cell cancer that has been found in the distal arm of 3q [79].

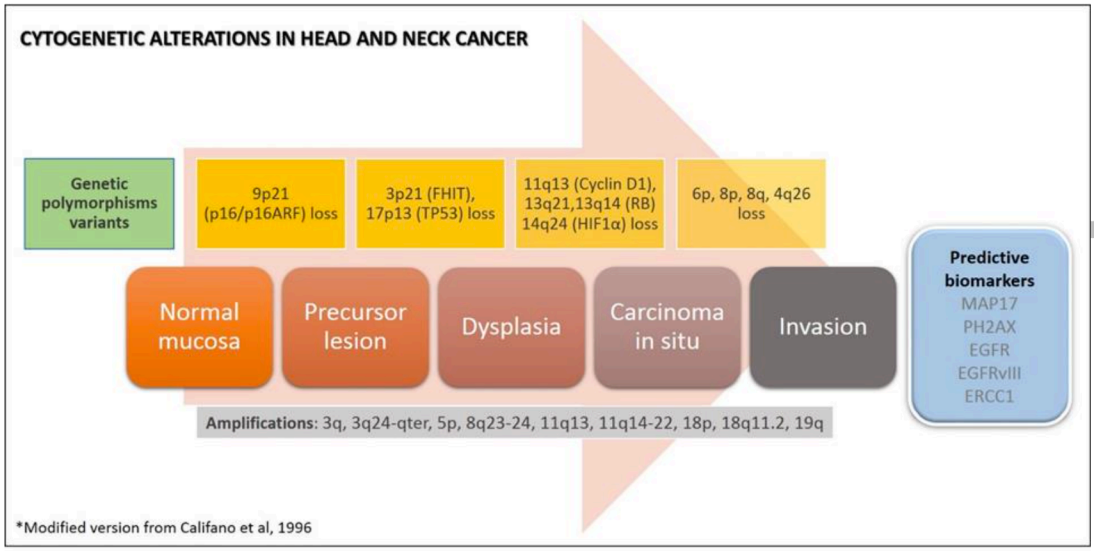


Figure 8. *Frequent cytogenetic alterations in head and neck cancer.*

Loss of chromosomes 3p, 5q, 8p, 9p, 18q and 21q are commonly identified as well, where loss of 18q could indicate poor prognosis tumours (80). The most commonly deleted region in HNC is located at chromosome 9p21-22 [81]. It occurs in the majority of invasive tumours and is present at a high frequency in early premalignant lesions, including dysplasia and carcinoma in situ [82]. P16 or cyclin-dependent kinase inhibitor 2 (CDKN2-) is the tumour suppressor gene contained

within this critically deleted region and is a potent inhibitor of cyclin D1/CDK4 [83]. However, p16 amplification is independent of cyclin D1 inactivation in HNC [84]. It is possible that a second tumour suppressor gene resides at 9p21. In the same INK4 loci, an alternative reading frame codifies for a very different unrelated protein, p14ARF, which regulates MDM2 and therefore p53. Introduction of p16 or p14ARF into head and neck cancer cell lines results in potent growth suppression [85]. Loss of heterozygosity (LOH) at the Retinoblastoma (Rb) locus in 13q14 has been found in around 14% of HNC, leading to Rb protein inactivation and tumour progression [86]. Rb LOH is correlated with altered pRB expression in endometrial and oesophageal cancer [87, 88] but same results could not be found for laryngeal cancer [89].

1.4.3. TP53

Mutations in P53 are one of the most frequent abnormalities in HNC and can be observed in severe dysplasia. TP53 mutations are found in 39-53% of HNC tumours and in 56.7% of laryngeal carcinomas [90, 91]. P53 mutations have been demonstrated to be related to poor survival in different publications by using microarray technology [90], immunohistochemistry (IHC) [92] or single-strand conformational polymorphism (SSCP) analysis followed by DNA sequencing. However, there is not necessarily correlation between IHC and SSCP results [91]; moreover, decrease in survival can vary if the mutation is disruptive or non-disruptive [90]. These controversies may explain differences as a prognostic factor found so far.

1.4.4. Notch

Inactivating mutations of Notch1 have been found in 10-15% of HNC, being the second most frequently mutated gene after TP53 [93,94]. Notch signalling pathway has been linked to multiple biological functions, such as regulation of self-renewal capacity, cell cycle exit, and survival. In HNC, several of the Notch family mutations encode inactivating mutations, suggesting a tumour suppressor function [95]. In a study that included 289 patients with laryngeal carcinoma Notch3 was associated with unfavourable disease-free survival and overall survival [96].

1.4.5. EGFR/EGFRvIII

The EGFR pathway is involved in cell transformation through autocrine overproduction of epidermal growth factor/transforming growth factor alpha (EGF/TGFá) and overexpression of EGFR by gene amplification or altered transcriptional mechanisms [97]. Production of TGFá and EGFR mRNA has been found in normal mucosa of patients at risk for a primary or secondary HNC suggesting these changes to be early markers of carcinogenesis [98].

In up to 90% of HNC, increased expression of EGFR is observed which is associated with advanced stage, poor survival and resistance to treatments [99, 100]. Similarly, EGFR overexpression in laryngeal carcinoma has also been linked to poor survival [101] and also as a predictive biomarker for radiotherapy treatment [102]. In a phase III trial comparing cisplatin with or without the monoclonal antibody cetuximab for metastatic/recurrent HNC patients, it was seen that doses of cetuximab may need to be adjusted for patients with very high levels of EGFR to achieve better response [103]. The EGFRvIII mutant variant has been found in up to 40% of HNC and it seems to contribute to cancer growth and resistance to EGFR targeting. EGFRvIII is seen in cells that overexpress wild-type EGFR suggesting that mutations are a later event caused by rapid proliferation induced by EGFR overexpression [104].

1.4.6. DNA damage repair biomarkers. MAP17 and pH2AX

As laryngeal cancer responds to treatments based on platinum and radiotherapy that result in DNA damage, biomarkers implicated in the nucleotide excision repair (NER) and the double strand breaks repair might have a significant role. DNA Double break streams (DBS) can be originated by drugs and ionizing radiation but also by increasing the levels of reactive oxygen species (ROS) [105] through MAP17 activation. MAP17 is a small 17 Kda non-glycosylated membrane protein overexpressed in carcinomas. It has been found present in adenoma and benign tumours, and is highly expressed in metastatic carcinoma. The expression is mainly driven at a transcriptional level either by promoter activation or demethylation. Expression of MAP17 in primary cells triggers senescence through p38, but in tumoral cells enhances the malignant capabilities of these cells, increasing proliferation, migration, resistance to apoptosis, etc. MAP17 expression increases the levels of ROS in cells which may account for some of the increased tumoral properties. In turn, a further increase of ROS might switch the balance towards apoptosis. Thus, MAP17 may increase the efficacy of therapies increasing ROS and therefore constitute a biomarker for better prognosis of these tumours. In cervix tumours treated with cisplatin and radiotherapy, high levels of MAP17 mark good survival of the patients. Therefore, MAP17 is not only a marker for stage and malignant status but also may be a marker of prognosis and response to therapies involving oxidative stress [106-108]. DNA DBS lead to activation of three kinases, ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR) and DNA-PK which phosphorylate γH2AX, a component of the histone octamer in nucleosomes. PH2AX is involved in recruiting DNA repair proteins in response to double-strand breaks (DSB) [108]. Therefore, it is considered as a biomarker of DNA damage, as the presence and magnitude of pH2AX is an indication of persistent, unrepaired DNA damage. pH2AX induction appears within minutes in cells after DNA damage and reaches maximum levels after 30 minutes. The repair process includes the phosphorylation of hundreds to thousands γH2AX surrounding the DSB site in order to form a

focus that open the chromatin structure and serve as a platform for the accumulation of factors involved in the DNA damage response [109]. The NER pathway guard the integrity of the genome by recognizing and removing DNA cross-links caused by cisplatin or radiation mainly driven by the excision repair cross-complementation group 1 (ERCC1) [110]. In locally advanced HNC low expression of ERCC1 was an independent predictor factor for prolonged in patients treated with cisplatin-based CCRT; however just 18% of patients had laryngeal carcinoma [111]. Furthermore, in a case-control study focused on laryngeal cancer, ERCC1 rs11615 and ERCC5 rs17655 polymorphisms were associated with increased risk of developing laryngeal cancer [112].

1.4.7. Vaccinia-related kinase-1 (VRK1) protein

VRK1 protein belongs to a family of three protein kinases implicated in regulation of cell proliferation by phosphorylation of p53 and cooperation with c-Jun and ATF2. VRK1 expression is activated by E2F and inhibited by p16 and Rb. In HNC, VRK1 could be a significant control mechanism of the cell cycle, particularly in G1-S phase [113].

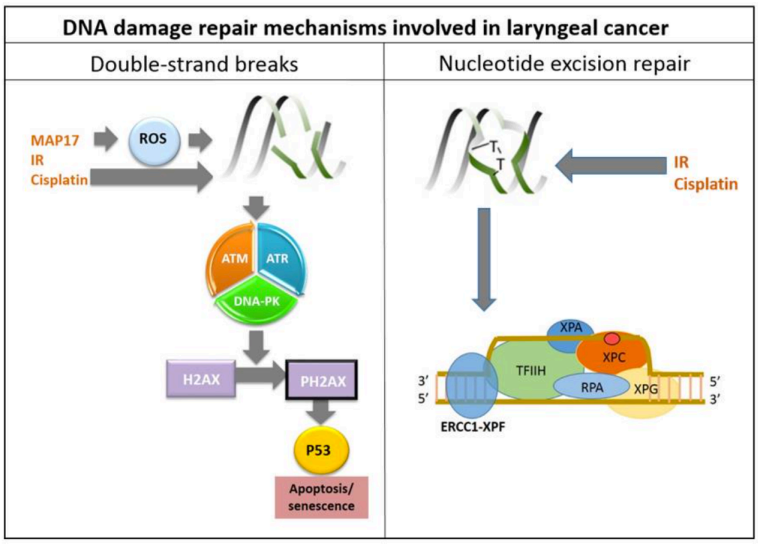


Figure 9. *Mechanism of response to DNA damage in laryngeal cancer. IR: ionizing radiation.*

1.4.8. STAT3 pathway

Stat3 (signal transducer and activator of transcription 3) is a transcription factor that responds to cytokines and growth factor receptor activation [114]. Constitutive activation of the pathway in response to deregulate upstream signals is commonly observed in diverse cancers including dead and neck and laryngeal tumours [115-117]. This constitutive activation of the pathway has been involved in proliferation and survival of tumours as well as resistance to chemo and radiotherapy

[116,117]. Recent works indicate that STAT signalling also contributes to therapy resistance by modulating also the microenvironment [118]. ERp57 (GRP58) is a chaperone that regulated proper folding of glycoproteins [119]. ERp57 is associated with tumour progression and has been described to modulate STAT 3 activity, thus regulating radioresistance in laryngeal cancer [120]. Accordingly, ERp57 has been described as a poor prognosis factor.

1.4.9. Micro RNAS

MicroRNAs (miRs) play important roles in many pathological alterations regulating important cellular and physiological processes such as cell proliferation, differentiation, metabolism, apoptosis, autophagy and intercellular communications [121, 122]. It has been considered that miRs regulate around 60% of genes in the human genome. The analysis of miR expression variation in Laryngeal cancer by a variety of techniques including broadly used miR microarrays and massive sequencing have shown a large variety of miRs deregulated in laryngeal cancer, many of them with diagnosis or prognosis value (reviewed in (123)) [124,125] (Table 4). These miRs behave as oncogenes (oncomirs) or tumour suppressors in laryngeal cancer according the effect of the target. However, this simplistic analysis is commonly more complicated since one miR can target several genes and several miRs can target the same gene thus providing some synergistic effects. Interestingly, several miRs have been associated with therapeutic resistance in laryngeal cancer, thus providing worse prognosis.

MIRNAS	TARGET	PROGNOSIS
UPREGULATED		
miR-16	Zyxin	ND
miR-19a	TIMP2	Poor survival, Lymph node metastasis
miR-21	BTG2	Poor survival, poor differentiation and Lymph node metastasis
miR-27a	PLK2	ND
miR-106b	RUNX3	Poor survival, poor differentiation and Lymph node metastasis
miR-129-5p	APC	ND
miR-155	SOCS1, STAT3	Poor differentiation and TNM stage
miR-1297	PTEN	ND
DOWNREGULATED		
miR-1	FN1	ND
miR-24	S100A8	ND

miR-144-3p	ETS-1	Poor prognosis
miR-34a	Survivin	Good prognosis
hsa-miR-34c	C-Met	ND
miR-126	Camsap1	ND
miR-139	CXCR4	ND
miR-203	ASAP1	Good survival , inverse to TNM and grade of differentiation.
miR-206	VEGF	Good survival , inverse to TNM and clinical stage.
miR-299-3p	hTERT	ND
miR-370	Fox-M1	ND
miR-519a	HuR, COX-2	ND
miR-874	HDAC1	ND

Table 4. *Summary of miRNAs relevant in laryngeal cancer. Adapted from Yu and Li, 2015 [10]. ND: not determined*

1.4.10. Tumour microenvironment. Immune-phenotypes

Most tumour cells express antigens that can mediate recognition by host CD8+ T cells. Cancers that are detected clinically must have evaded antitumor immune responses to grow progressively. HNC analysis of tumour microenvironment has revealed the presence of two major subsets of tumours with distinct mechanisms of resistance to immune-mediated destruction.

The inflamed (and mesenchymal) phenotype present in a group of HNC tumours show prominent tumour infiltration by CD8(+) lymphocytes and a broad chemokine profile. This is independent of HPV status. Immune resistance occurs after T-cell migration into the tumour site, implicating the effect of negative immune regulators. On the other hand, the non-inflamed tumours are devoid of T cells and other indicators of innate immunity, such as chemokines. Immune failure is attributed to poor effector T-cell trafficking, as required factors are absent. These two major phenotypes of tumour microenvironment may require distinct immunotherapeutic interventions for maximal therapeutic effect [(126)].

1.5 LARYNGEAL CANCER TREATMENT

1.5.1. Preservation approaches

Organ preservation treatments for laryngeal cancer patients depend on whether the tumour is presented in early stages (I and II) or advanced locoregional disease (stage III/IV). In general, early stages are treated with either primary surgery or definitive radiotherapy (RT), while advanced stages require a multimodal approach.

Early stage disease (Stages I and II)

Total laryngectomy was the gold standard treatment by the 1980s with the subsequent loss of speech and airway patency. Because of the significant quality of life decreasing of these patients, partial laryngectomies and endoscopic laser surgeries were initiated in the 90´s in order to preserve laryngeal function. Furthermore, 95% local control rates have been described in patients with T3 and T4 glottic and supraglottic tumors who underwent supracricoid laryngectomy, with an associated improvement of quality of life [127,128].

Later, radiotherapy was given to early stages. Although surgery and radiotherapy have never been compared in a randomized trial, both have been accepted to have similar effectiveness. This is based on one-arm prospective trials and prospective case-control series. In one of these series published in 2002, 31% locoregional control was reported for T3 laryngeal cancer with an organ preservation rate of approximately 50% [129]. Alternative radiotherapy schedules were studied such as the hyperfractionated or the accelerated radiotherapies. Both techniques showed improved laryngectomy free-survival, and increased locoregional control compared to standard radiotherapy without increasing toxicity. However, none of them could show a benefit in terms of survival. The RTOG 90-03 phase III trial confirmed those results and demonstrates a better local control rate with hyperfractionated and accelerated radiotherapy compared to standard treatment (55% vs 45%) [130].

Locally advanced (Stages III and IVA/B)

Although functional organ sparing approaches permit larynx preservation, they do not provide a survival advantage over total laryngectomy [131]. Three sparing approaches are accepted: RT, bio or chemotherapy with concomitant radiotherapy (B/CT RT) and induction CT (ICT) followed by RT with or without B/CT.

CTRT with concurrent cisplatin showed higher preservation rates compared to other two arms with RT alone or induction cisplatin plus fluorouracil followed by RT (88% vs 70% and 75%, respectively) with similar two and five-year survival [132]. Later, a 10-year follow-up publication

confirmed that the arms that included ICT improved laryngectomy-free survival (LFS). Contrary to preservation rates, LFS includes not just the need of salvage laryngectomy but also speech and swallowing quality. It is, therefore, more similar to what we currently understand as larynx preservation [133]. A subsequent meta-analysis for locally advanced larynx cancer found that adding CT concomitant with RT led to a benefit of 6.5% absolute improvement in 5-year OS [134]. In a phase III trial, patients with advanced head and neck cancer who received combination treatment with RT and cetuximab, demonstrated a statistically significant advantage with respect to locoregional control (LRC) and survival compared to patients treated with radiation alone [135]. A subset analysis of the patients with hypopharyngeal and laryngeal tumors showed a preservation hazard ratio (HR) of 0.62 in the cetuximab arm but this was not statistically significant [136]. The optimal dose of cisplatin during RT remains still unclear. This was studied as a subset analysis of the RTOG 0129 phase III trial, which compared accelerated concomitant boost vs standard RT fractionation. Receiving one cycle of 100 mg/m2 cisplatin was associated with worse OS, PFS and locoregional failure compared to two and three cycles. The third dose of cisplatin had no impact on OS or PFS compared with two cycles, but was associated with better LRC rate [137]. A meta-analysis presented in 2011 also suggested that there could be a dose/efficacy relation for concomitant cisplatin total dose. In this analysis, no advantage in OS was observed between CRT with cisplatin total dose <150mg/ m2 and RT alone. No difference in OS was observed between cisplatin high dose (300mg/m2) (HR 0.59, 95% CI 0.46-0.74) and cisplatin <300mg/m2 plus 5-fluorouracil (HR 0.59, 95% CI 0.45- 0.77) when compared to RT alone [138]. Finally, a systematic review showed that in six definitive radiotherapy phase III trials there was a statistically significant association between cumulative cisplatin dose, and overall survival benefit for higher doses [139]. In summary, two or three courses of three-weekly cisplatin could be considered the optimal dose for concurrent CTRT and equivalent doses of carboplatin have also been accepted by expert panels.

Regarding ICT, the Veterans study demonstrated 64% preservation larynx rate without worsening survival in the ICT-RT arm compared with surgery-RT [63]. More efficacious induction regimens were further developed and the docetaxel, cisplatin, and 5- Fluorouracil (DCF) schedule became the standard treatment, preserving the larynx 15% more than cisplatin and 5-Fluorouracil [140]. DCF induction followed by radical RT with concomitant cetuximab or cisplatin showed similar larynx preservation rates in both arms in a phase II randomized trial. Endpoints were evaluated on the randomly assigned population only, which represented 76% of the patients included in the trial so they were inflated. None of the arms could show any substantial benefit compared with the GORTEC 2000-01 trial, and therefore there is still not evidence enough for sequential therapy in this setting [141] (Table 5).

1.5.2 Metastatic disease (stage IVC)

Despite treatment progress, locoregional recurrences and distant metastases occur in 20-30% of patients with SSCHN, and just few of them benefit from salvage surgery or re-irradiation. Treatment options for metastatic disease include supportive care, single agent or a chemotherapy combination with or without targeted agents [142]. Prognostic factors of long-term survivors in metastatic SCCHN patients treated with platinum-based chemotherapy were identified in the E1395 and E1393 randomized trials of the Eastern Cooperative Oncology Group (ECOG) and include: tumour cell differentiation, ECOG performance status (PS), weight loss, location of the primary tumour and prior radiotherapy [143].

The EXTREME trial showed that the combination of platinum-5Fluorouracil and cetuximab as first-line treatment in SSCHN improved OS, PFS and response rate with no decreased quality of life compared to the same schedule without the monoclonal antibody. Therefore, it has become the standard of treatment for patients with good PS [144]. Less intensive schedules include de combination of cisplatin-paclitaxel or cisplatin-5Fluorouracil, as they have been shown to achieve similar overall survival [145]. Other regimens such as cisplatin plus cetuximab improved response rate in a phase III trial but with no benefit of PFS which was the primary end-point [146]. Single agent activity remains poor but it may be an alternative when other options are exhausted. Classical drugs as methotrexate, cisplatin, 5-fluorouracil (5-FU) and bleomyin have shown responses of short duration (3-5 months) and 15-30% tumour reduction [142]. Of the more recent agents, taxanes (docetaxel and paclitaxel) improved response rates up to 43% in platinum resistant patients [147]. Furthermore, afatinib is an irreversible ERBB family blocker with significant results in the second-line setting. In a recent phase III trial it was compared to methotrexate in patients that had progressed to platinum-based regimens (including also cetuximab) and the primary end-point PFS was superior (2.6m vs 1.7m, p=0.03). However, G3/4 toxicities were also higher in the afatinib arm [148]. Finally, different immunotherapies agents are being studied for HNC. Of them, nivolumab is the most advanced as the final results of the phase III trial have been recently published. Patients after relapse or progression within 6 months of platinum therapy were randomized to biweekly nivolumab vs dealer´s choice. The primary outcome was overall survival which was 7.5 vs 5.1 m with a 95% IC. Moreover, at the time of analysis, 17.4% were still receiving nivolumab 2.7% were still receiving standard therapy. Although there is still a need for biomarker validation and patient selection, nivolumab could be considered as a new standard of care for second line treatment HNC [149].

Trial	Phase, patients	Design	Inclusion criteria	Primary end point	Primary end point result	Secondary end points	Statistics	Others
Veterans 1991	III, 332P	CFx3->RT (if response to CF) vs S->RT	III/IV glottic or supraglotic	Preservation rate	Preservation 64% vs 0%	31% CR, 54% PR. 68% 2-year OS in both arms	All randomized P included	More local recurrences with fewer distant mets in the CF arm. Latter analysis showed T4 to have better outcomes with surgery.*
Forastiere, RTOG 91-11 2003	III, 518P	3 arms: CF->RT vs cis-RT vs RT	III/IV glottic or supraglottic **	2-y LFS	Cis-RT 88% CF->RT 75, RT 70%	LRC cis-RT 78%, CF->RT 61%, RT 56%. Similar OS rates	Designed to test if cis-RT or RT resulted in higher rates of preservation than CF->RT. Detect 15% difference, type I error=0.05, type II=0.20.	
Forastiere, RTOG 91-11 2013	Previous trial 10-y update	3 arms: CF->RT vs cis-RT vs RT	III/IV glottic or supraglottic **	10-y LFS	Cis-RT 23.5%, CF->RT 28.9%, RT 17.2% (cis-RT vs CF->RT HR=1.05, p.68)	Laryngeal preservation: cis-RT vs CF->RT HR=0.58, p.005. LRC: cis-RT vs CF->RT HR=0.66, p.006		The benefit of cis-RT for LFS is not maintained, but secondary end-points are. No collected information about gastrostomy tube need. 43% CTRT P had severe late toxicity ***
Bonner 2006	III, 424P	RT with or without cetuxi	III/V oropharynx, hypopharynx, and larynx	LRC	Cetuxi-RT vs RT HR=0.68, p.005. (2y= 50 vs 41 %, p.005)	cetuxi-RT OS HR=0.74, p.03	Planned 1y-LRC RT 44%, cetuxi-RT 57%. 90% power, 5% two sided LRC	Major benefit for patients with oropharynx cancer. OS subset analysis for laryngeal tumours revealed no differences between arms (p=.87)
Bonner 2010	Previous trial 5-y update	RT with or without cetuxi	III/IV oropharynx, hypopharynx, and larynx	LRC/OS	5-year LRC and DFS were not reported. OS in the cetuxi-RT vs RT HR 0.73, p=0.018			P with G2-4 cetuxi-induced acneiform rash had better OS that patients with mild rash.
Vermorken, 2007	III, 358P	DCF vs CFx4 cycles-> RT if no PD	III/IV SCCHN (with no M1)	PFS	DCF group HR 0.72, p=0.007	Reduction in the risk of death 27%	90% power to detect 15% differences in the 1-y survival rate	Ciprofloxacin as primary prophylaxis. G-CSF as secondary.
Pignon, 2009	Meta-Analysis, 16485P	Locoregional treatment +/- CT.	Non-metastatic HCN	OS	DFS,LRC, and distant failure	Absolute benefit for CT 4.5% at 5 years. For concomitant trials, HR 0.81 p.0.0 and absolute benefit 6.5% at 5 years. Concurrent CDDP was identified as the most effective agent.		
Pointreau, GORTEC 2000-01 2009	III, 213P	CF vs DCFx3-> CTRT ****	III/IV larynx/ hypopharynx	Preservation rate	DCF 70.3% vs PF 57.5%, p=0.03	ORR DCF 80.0% vs PF 59.2% p=0.002	70% power to detect 15% improvement preservation.	Ciprofloxacin as primary prophylaxis. G-CSF as secondary.
Koutcher, 2011	Retrospec tive, 174P	Cis-RT vs cetuxi-RT	III/IV SCCHN	Cis-RT superior for 2-y locoregional failure (5.7% *v* 39.9%; *p*.001), failure-free survival (87.4% *v* 44.5%; *p*.001), and OS (92.8% *v* 66.6%; *p*.001).				Lack of HPV data. Possibly biased as P in cetuxi-RT were older and with renal impairment.
Lefebvre, Tremplin 2013	II, 153P	DCFx3-> cetuxi-RT or cis-RT	Locally advanced larynx/hypopharynx	3-m preservation	No differences: Cis-RT 95% vs cetuxi-RT 93%.	Preservation 87% cis-RT vs 82% cetuxi-RT; 18-m OS: 92% vs 89%.	Studied on randomly assigned population (76%) so inflated. 90% power; 5% type I error. No stratification.	Local recurrence: Cetuxi 21% vs Cis 8% (p=0.08). None of the arms could show any substantial benefit compared with the GORTEC 2000-01 trial.

Table 5. Summary of the most significant clinical trials involving locally advanced laryngeal cancer treatment. P= patients. SCCHN: squamous cell carcinoma of the head and neck. CF: cisplatin, 5- Fluorouracil. RT: radiotherapy. S: surgery. CR: complete response. PR: partial response. PD: progression disease. OS: overall survival. Mets: metastases. Cis: cisplatin. Y: years. M: months. DFS: disease-free survival. LFS: laryngectomy-free survival. LCR: locoregional control. Cetuxi: cetuximab. PFS: progression-free survival. DCF: docetaxel, cisplatin, 5- Fluorouracil. ORR: overall response rate. *Wolf GT. Reexamining the treatment of advanced laryngeal cancer: the VA laryngeal cancer study revisited. Head Neck 2010;32:7-14.** T1 and high-volume T4 were excluded: invasion >1 cm into de base of tongue or penetration through cartilage. *** Chemotherapy (cisplatin, carboplatin, and 5-fluorouracil or a combination of two drugs) during radiotherapy was allowed for all patients who were treated at the same institute, according to its practice.

2.1. HYPOTHESIS

There are different organ preservation treatments available for patients with non-metastatic laryngeal cancer. Because these regimens can maintain laryngeal function, are generally preferred to surgery. On the other hand, no benefit on survival has been determined with preservation approaches, and they confer significant acute and late toxicities. Furthermore, around 30-40% of patients relapse or lead to an incompetent larynx and might end having surgery [63].

The high rate of toxicities and non-improved survival with preservation approaches lead to the need for biomarker development. Predictive larynx biomarkers would facilitate pre-treatment identification of those patients who are unlikely going to be cured by radiation-based therapy. By managing these patients with surgery rather than a preservation approach, local disease control and possibly survival could potentially be optimized and unnecessary treatment related morbidities from unsuccessful larynx treatments could be avoided.

2.2. GOAL

With this study we aim to find clinical and/or biological biomarkers that could be related with a better prognosis and potentially be used as predictive biomarkers for pre-treatment patient selection.

2.3. ENDPOINTS

2.3.1 Primary endpoint

To determine whether molecular biomarkers such as MAP17 and PH2AX are associated to survival in patients with non-metastatic laryngeal cancer treated with preservation approaches.

2.3.2 Secondary endpoints

-To determine clinical factors implicated in survival and laryngeal preservation.

-To determine whether molecular biomarkers are associated to laryngeal preservation.

-To study the correlation between clinical and biological biomarkers and their relationship with survival.

-To study the role of the DDR pathway in laryngeal pathway and patient´s survival.

3.1 PATIENT´S CHARACTERISTICS AND TREATMENT

We evaluated 65 patients with larynx cancer from August 2005 to February 2014. However, out of the 65 tumoral samples, only 53 of them could be studied. All samples were obtained from diagnostic biopsies before treatment. All patients completed the informed consent form and the project was approved by the local ethical committee at the Hospital Universitario Virgen del Rocío (HUVR) (PI13/059). Patients received treatment in our institution but tumour samples were obtained from four different national hospitals where the diagnosis was made. Eligibility criteria for treatment preservation include patients with stage II-IV laryngeal tumours that had no contraindication for CT or RT, significant cartilage destruction, or more than 2 cm tumoral invasion of the base of the tongue. TNM Staging System (7th ed., 2010) was used for tumour classification [27]. Patients were mainly male (94%) with squamous cell carcinoma and good general condition. Tumours were more frequently localized in the supraglottic (60%) and 75.5% were stage III. Approximately one third of patients required pre-treatment tracheotomy. Selected organ preservation therapies were B/CTRT (75%), RT (14%), or ICT-B/CTRT (9%). Cisplatin 100 mg per square meter (m^2) on days 1, 22, and 43 (74%) was most commonly used, followed by weekly cisplatin 40 mg/m^2 (11%), and monoclonal antibody cetuximab (11%) (Table 6).

Characteristics	**No.**	**%**
Mean age	62 years	
Male	61	94
Squamous cell carcinoma	65	100
Cigarette smoking		
Current smokers	44	68
Former smokers	19	29
Never smokers	2	3
Smokers of ≥ 10 pack-years	60	92
Regular alcohol intake	46	71
PS 0-1	62	95
Pretreatment tracheotomy	21	32
Localization		
Supraglottic	39	60
Glottic	24	37
Subglottic	2	3
TNM Staging		
II	6	9
III	49	75.5
IV	10	15.5
Treatment approach		
Surgery	1	2
Radiotherapy	9	14
B/CTRT	49	75
ICT-B/CTRT	6	9

PS: Performance status-ECOG; B/CTRT: bio/chemoradiotherapy; ICT-B/CTRT: induction chemotherapy followed by bio/ chemoradiotherapy.

Table 6. *Population characteristics and treatment.*

3.2 TISSUE ACQUIREMENT AND PREPARATION

Formalin-fixed, paraffin-embedded tissue sections from 65 laryngeal carcinomas were selected with the collaboration of the Andalusian Health Care Biological Resource Centre. Histological characterization of all samples was done by Hematoxylin and Eosin staining, followed by immunohistochemistry (IHQ) analysis of tissue microarrays (TMA).

3.2.1 Immunohistochemistry

Three-micrometer slices were sectioned from the TMA block and applied to coated, immunochemistry slides (DAKO, Glostrup, Denmark). The slides were baked overnight in a 56°C oven, deparafinized in xylene for 20 min, rehydrated through a graded ethanol series and washed with PBS. A heat-induced epitope retrieval step was performed by heating a slide in a solution of sodium citrate buffer pH 6.5 for 2 min in a conventional pressure cooker. After heating, the slides were incubated with proteinase K for 10 min and rinsed in cool running water for 5 min. Endogenous peroxide activity was quenched with 1.5% hydrogen peroxide (DAKO) in methanol for 10 minutes, and incubation with the primary antibodies was performed for 40 min. Selected antibodies were: anti-gamma H2A.X (phospho S139) antibody (ab11174 from Abcam), anti-p53: p53 FL 393 (sc-6243 from Santa Cruz), anti-MAP17 (1:4) [29-33], anti-SGLT1 (Abcam #14685), ki67 (clone MIB-1; DAKO, Agilent technologies, United States), AKT-p (phosphorylated S473-AKT1, Epitomics), and p44/p42 MAPK (Erk1/2) Rabbit mAb 1:1000 (Cell Signaling 137F5). After incubation, immunodetection was performed with the EnVision (DAKO, Glostrup, Denmark) visualization system using diaminobenzidinechromogen as the substrate, according to the manufacturer's instructions. Immunostaining was performed in a TechMate 500 automatic immunostaining device (DAKO) and measured through a double- blind visual assessment using microscopic observation according to the anatomopathological experience of pathologists. Sample scoring was performed by semiquantitative microscopic analysis, considering the number of stained cells (percentage of positive cells) and signal intensity (levels 1, 2, or 3).

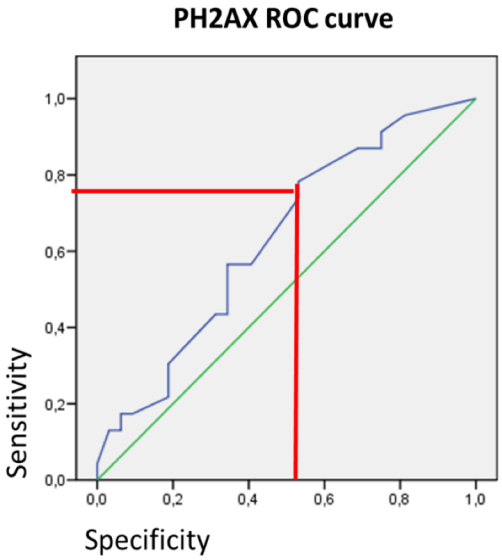
3.3. CELL CULTURE FOR IN VIVO RADIATION TREATMENT

Hela malignant cervical tumour cells were obtained from the European Collection of Cell Cultures (ECACC) human cell line repository and maintained in Dulbeccós modi ed Eaglés medium (Sigma) containing 10% fetal bovine serum (Sigma), penicillin, streptomycin and fungizone. MAP17 full-length cDNA was cloned into pBabepuro and mass culture generated by stable gene transfer in Hela cells. After selection with 2 ìg/ML puromycin, mass cultures were used for the study. As a control, Hela cells were transfected with pBabepuro alone and selected. Cells were irradiated using Costar 24 well cell culture plates (Corning Incorporated, NY USA).

To simulate actual radiobiological experimental conditions, each well was filled with culture medium. The plate dimensions were 12.5 x 8.5 cm. The inner diameter of the well was 16 mm and the distance between the centers of two neighboring wells was 20 mm. Plates were positioned inside a water-equivalent device, specifically designed to fit the plate. This device measures 16 x 16 x 2 cm, and is placed inside the IBA BodyPhantom (IBA Dosimetry GbmH, Schwarzenbruck, Germany) at a depth of 6 cm. Simulation was performed using a Toshiba Aquilion CT scanner (Toshiba Corporation, Japan). CT images were exported to the treatment planning system Philips Pinnacle V9.2 (Philips Radiation Oncology Systems, Madison, WI). Five plans were designed to deliver uniform doses of 0.1 Gray (Gy), 0.3 Gy, 1 Gy, 3 Gy and 10 Gy using static beams of 24 x18 cm. To verify the dose within every well, we delineated 24 regions of interest (ROI) that had a diameter of 16 mm. The ROI was estimated at the bottom of the wells and 5mm upwards. The dose delivered to the cells was verified with the IBA Compass system (IBA Dosimetry GbmH, Schwarzenbruck, Germany). Differences between the prescribed dose and the dose received were within 3%. The irradiation was delivered using 6 megaelectronvolts (MV) photon beams from an Elekta Synergy Linac (Elekta Oncology System, Ltd, Crawley, UK) with a dose rate of 500 mu/min.

3.4. STATISTICAL ANALYSIS AND DEFINITIONS

Kaplan-Meier method was used for survival analysis, using Cox Proportional Hazards model to adjust for the explanatory variables, obtain the p-values and estimate the hazard ratios (HR). Multivariate logistic regression was used to obtain odds ratio (OR) and CI 95%. Pearson's correlation measured dependence between quantitative variables. A receiver operating characteristic (ROC) curve was performed to assess the biomarker cut-off point (two-year OS for pH2AX and three-year OS for MAP17), which was confirmed using the optimal Youden index-based point. In addition, the log-rank test was used to compare survival distributions. Categorical data were studied with contingency tables that included Chi-square statistics. Calculations were performed using SPSS 15.0 software. OS has been defined as the length of time from diagnosis until the last medical record. Locoregional control (LRC) was measured as length of time from diagnosis until the relapse or last medical record, in those patients who did not develop distant metastases or died due to different causes than the tumour. For laryngoesophageal dysfunction-free survival (LDS) we adopted Lefebvre Larynx Preservation Consensus Panel that included as endpoint events: death, local relapse, total or partial laryngectomy, tracheotomy at two or more years, or the presence of a feeding tube at two or more years [(150)].



	Sensitivity	1 - Specificity
-1,0000	1,000	1,000
,5000	,957	,813
1,5000	,913	,750
2,2500	,870	,750
3,7500	,870	,688
5,2500	**,783**	**,531**
7,7500	,739	,531
10,5000	,565	,406
11,7500	,565	,375
13,7500	,565	,344
15,5000	,435	,344
18,0000	,435	,313
21,2500	,304	,188
23,7500	,261	,188
27,5000	,217	,188
32,5000	,174	,094
37,5000	,174	,063
45,0000	,130	,063
55,0000	,130	,031
65,0000	,043	,000
71,0000	,000	,000

Figure 10. *Example of ROC curve to determine the cut-off point for pH2AX.*

4.1. MAP17 (PDZKIP1) AS A NOVEL PROGNOSTIC BIOMARKER FOR LARYNGEAL CANCER

MAP17 is a small 17 Kda membrane protein present in carcinoma, but also in adenoma and benign tumours, and is highly expressed in metastatic carcinoma. Its expression correlates with staging and malignant status of the tumour. MAP17 expression is associated with an SGLT-dependent ROS increase that acts as a second messenger enhancing tumorigenesis. While a mild increase in ROS has been shown to activate signalling cascades that upregulate tumorigenic processes, further ROS increases lead to a potentially toxic cellular environment and programmed cell death. The hypothesis is that tumours expressing high levels of ROS producing MAP17 and SGLT1 proteins can benefit from therapies such as cisplatin or radiotherapy that increase oxidative stress and could sensitize them to cell death. In this work we have explored the relevance of the presence of MAP17 in larynx tumours where primary response is mainly achieved by treatments with radiotherapy and platinum compounds or other radiosensitizers.

4.1.1. Clinical cohort description

Lymph node metastases were significantly associated with decreased OS (N0 63.1 m, N1 38.6 and N2 22.2 m, p=0.019) while tumour local extension impacted LDS negatively (T4 extension 7.3 m vs. 47.1m non T4 extension, p=0.003). Besides, patients who required pretreatment tracheotomy had significantly worse LDS (54.3 vs. 18.9 months, p=0.001). The two-year cumulative proportion of patients with larynx preservation and OS were 57% and 76% respectively. Besides, locoregional control rate at two years was 60%, so similar to previously reported in the literature (section 1.5.1).

4.1.1. MAP17 expression in larynx tumour samples

Out of 65 samples, only 58 were analysed for MAP17 expression, either due to technical problems or because they did not contain any tumour cellularity. Out of the 58 samples, 46 (79%) were positives for MAP17 expression (Figure 11 A, B and C) and there was a trend showing higher levels of MAP17 in advanced grades of the tumour (Figure 11 D), although in this case, probably due to the low number of cases, it was not statistically significant. Surrounding normal tissue did not express MAP17 or expressed very low levels. We also analysed other markers for proliferation such as KI67 or the activated form of ERK (phosphorylated ERK, ERK-p), or apoptosis such as mutant p53 or activated AKT (phosphorylated AKT, AKT-p). Our cohort showed a percentage of samples positive for KI67, mutant p53, ERK-p or AKT-p, but these groups did not show correlation with MAP17 levels (Figure 12). However, KI67 positivity

showed statistically significant correlation with OS (Table 7). No correlation of MAP17 was observed with clinical parameters such as tumour localization, smoking habit, alcohol consumption, tumoral stage, pre-treatment tracheotomy and development of acute toxicities during chemoradiotherapy.

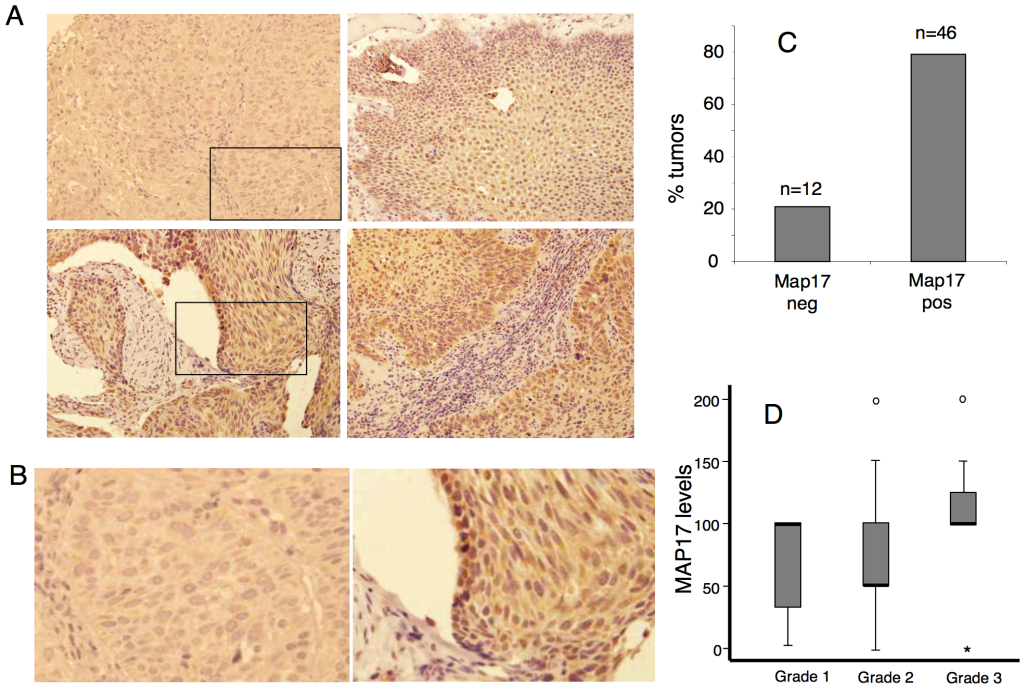


Figure 11. *MAP17 overexpression in larynx tumours. A) Representative images of MAP17 immunostaining are shown for different larynx tumours. B) High magnification of M17 positive and negative tumours. The picture shows a magnification of the inset of figure A. C) A graph is shown representing the percentage of laryngeal tumours with dichotomous MAP17 levels. The score for positive tumours were >62. D) The distribution of the MAP17 expression levels among different grades of larynx tumours is shown. The MAP17 levels (score) refers to maximum levels (0–2) scored by the percentage of cells (0–100). The normalized levels were obtained by multiplying the percentage of cells by the level of intensity observed. Anova test was performed to establish the statistical association between MAP17 protein levels and the grade of the tumour ($p<0,05$).*

	Laryngoesophageal dysfunction-free survival			Overall Survival		
Factors	**HR**	**p-value**	**CI**	**HR**	**p-value**	**CI**
Ki67	1.001	0.938	0.976-1.027	1.053	0.050	1.000-1.108
P53	0.982	0.209	0.956-1.010	1.016	0.477	0.972-1.062
SGLT	1.007	0.238	0.995-1.019	0.993	0.497	0.972-1.014
MAP17 (low levels)	32.66	0.001	4.352-245.1	21.73	0.010	2.071-228.1
PS=2	13.99	0.019	1.534-127.7	4.162	0.446	0.106-162.8
Pret. Tracheo.	1.476	0.431	0.560-3.890	1.849	0.505	0.304-11.26
RT	0.161	0.258	0.007-3.804	0.042	0.161	0.000-3.549
Bio/CTRT	0.046	0.080	0.001-1.452	0.109	0.447	0.000-33.00
ICT->bio/CTRT	0.047	0.101	0.001-1.805	0.014	0.169	0.000-6.054
Stage II	0.224	0.202	0.023-2.228	0.402	0.703	0.004-43.73
Stage III	0.636	0.500	0.171-2.367	0.048	0.016	0.004-0.569

Pret. Tracheo: pretreatment tracheotomy required. PS: ECOG-performance status. B/CTRT: bio/chemoradiotherapy; ICT-B/CTRT: induction chemotherapy followed by bio/chemoradiotherapy.

Table 7. *Laryngoesophageal dysfunction-free survival (LDs) and overall survival (Os) multivariate analysis of laryngeal cancer patients treated with preservation approaches. Pret. Tracheo.: pretreatment tracheotomy required. PS: ECOG-performance status. B/CTRT: bio/chemoradiotherapy; ICT-B/CTRT: induction chemotherapy followed by bio/chemoradiotherapy.*

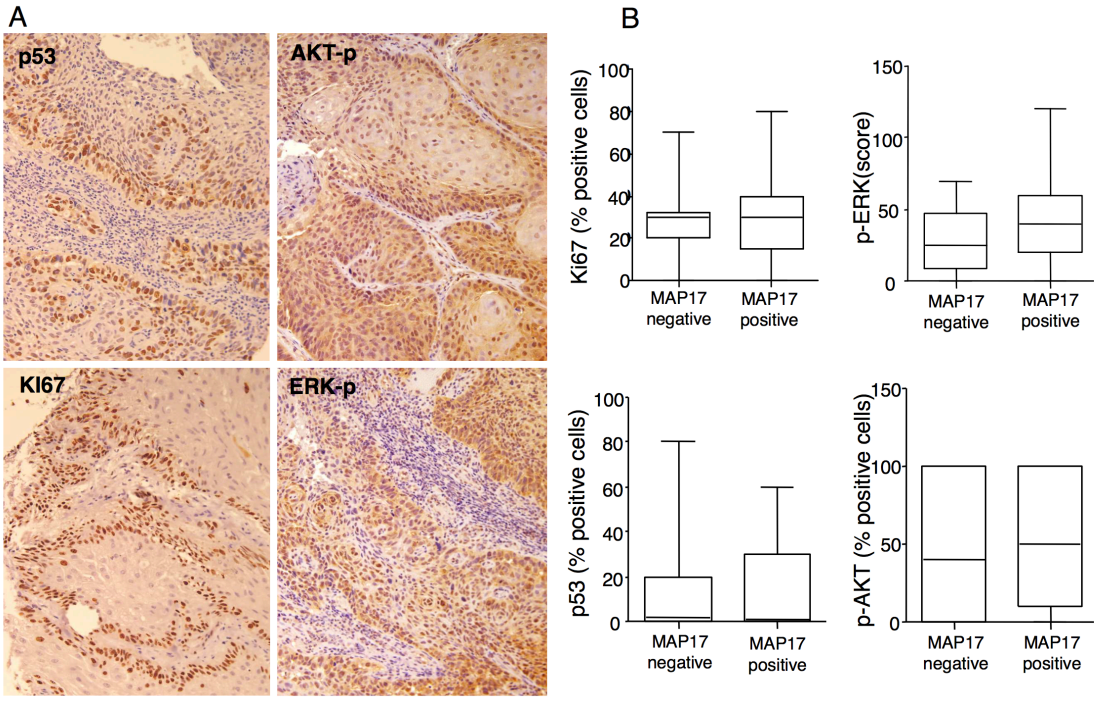


Figure 12. *p53, Ki67, p-ErK or p-AKt do not show correlation with MAP17 expression in larynx tumours. A) Representative images of p53, Ki67, p-ERK or p-AKT immunostainings are shown for larynx tumours. B) Graphs showing lack of correlation between these proliferative or antiapoptotic markers and MAP17 expression.*

4.1.2. SGLT1 overexpression in human larynx tumours correlates with MAP17 levels

Previous results indicated that MAP17-dependent tumorigenic properties depend on the indirect activation of ROS by SGLT1 transport and that there is a correlation between the expressions of both markers in cervix tumours [107]. Therefore, we measured SGLT1 expression levels in the same cohort of larynx tumour samples. We found that some tumours showed positive SLGT1 staining, with approximately 40% tumours being positive for SLGT1 (Figure 13 A, B, C and D). However, only a few samples showed very high staining levels. The distribution of the SGLT1-positive tumours among the different larynx tumours showed a clear correlation with MAP17 expression (Figure 13 E, F and G). Pearson indicator expressed a positive significant correlation between MAP17 and SGLT (P=0.3, p=0.022).

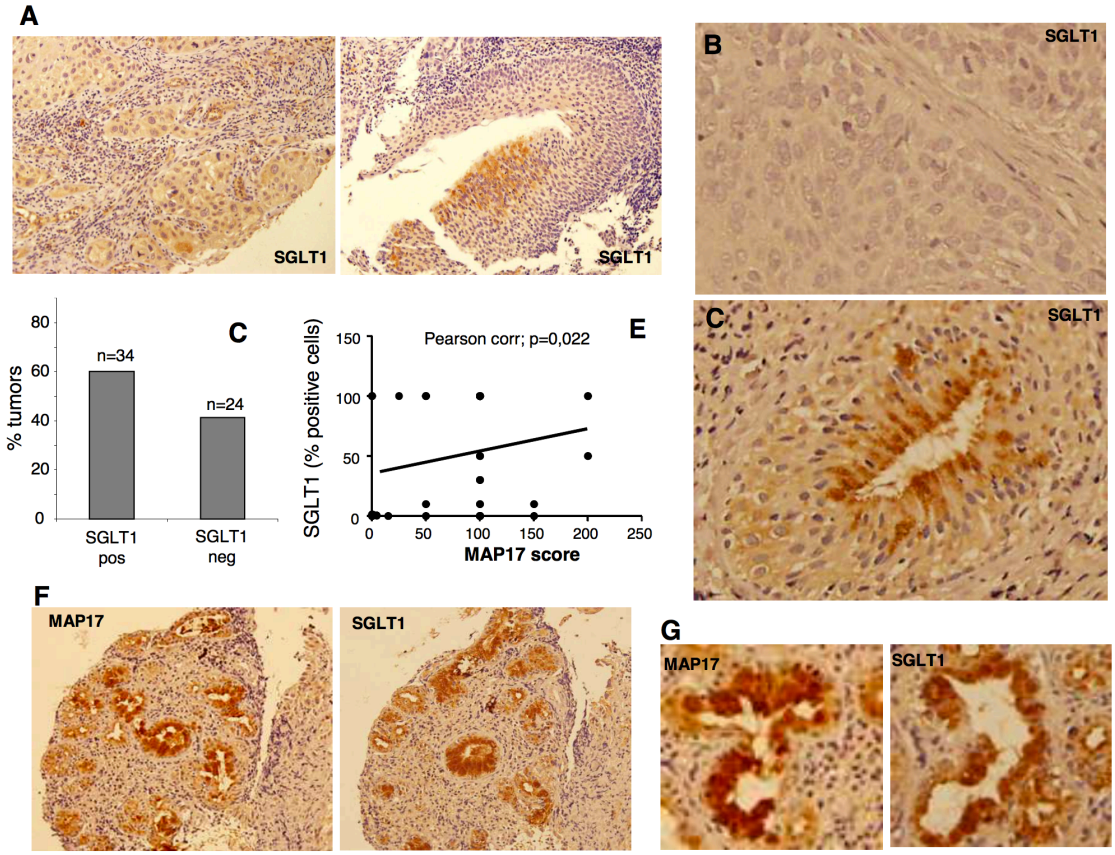


Figure 13. *SGLT1 overexpression in larynx tumours. A) Representative images are shown of SGLT1 immunostaining of different larynx tumours. B) High magnification of SGLT1 positive and C) Negative tumours. D) Graph representing the percentage of larynx tumours positive or negative for SGLT1 expression. E) Graph representing the correlation between MAP17 and SGLT1 expression in each tumour. The statistical analysis was performed by Pearson correlation (p=0,0022). F) Samples from one patient showing clear correlation between the expression of MAP17 and SGLT1. G) High magnification of samples from one patient showing clear correlation between the expression of MAP17 and SGLT1.*

4.1.3. MAP17 as predictive biomarker for laryngeal cancer

The high MAP17 group correlated in this study with better OS, LDS and LRC. When MAP17 was measured as a continuous variable, multivariate Cox model demonstrated that higher rates of MAP17 levels correlated with improved OS (HR 0.98, p= 0.001). Nevertheless, this could not be confirmed for LDS (HR 0.99, p=0.8), probably due to the limited number of cases. In order to distinguish a cut-off point for MAP17 levels a ROC curve was performed and punctuation of 62 score chosen. When measured as a dichotomous variable, high-MAP17 was related with increased OS, LDS, and LRC. A difference of 35.3 months was observed between high-MAP17 levels (67 months) and low-MAP17 levels (31.7 m) in the Kaplan- Meier model (IC 95%; p<0.001) (Figure 14) and the HR estimator for high-MAP17 was 0.78, p=0.002 in the multivariate analysis. Regarding LDS, high-MAP17 showed a survival benefit of 13.1m (47.6 m vs. 34.5 m, p=0.002) with a HR 0.14, p=0.003 in multivariate analysis. The effect of MAP17 high levels on the improved survival was significant after controlling for other variables: P53, Ki67, SGLT, PS, TNM, pretreatment tracheotomy and treatment received as shown in table 7. Regarding LRC, patients with high-MAP17 showed to have better outcomes than low-MAP17 (53.9 m vs 44.5 m, p=0.016) and the results were confirmed in the multivariate model (p=0.045). However, although MAP17 correlated with SGLT in the Pearson model (Figure 13E), SGLT by itself did not show statistically significant correlation with OS or LDS. Moreover, the association of high-MAP17 and high-SGLT show improved OS than MAP17 alone (72.4 m vs 42m, p=0,028) (Figure 14D). These data confirm that MAP17 alone, or preferably combined with SGLT1, is a good prognostic marker for survival in patients with larynx cancer treated with B/ CTRT.

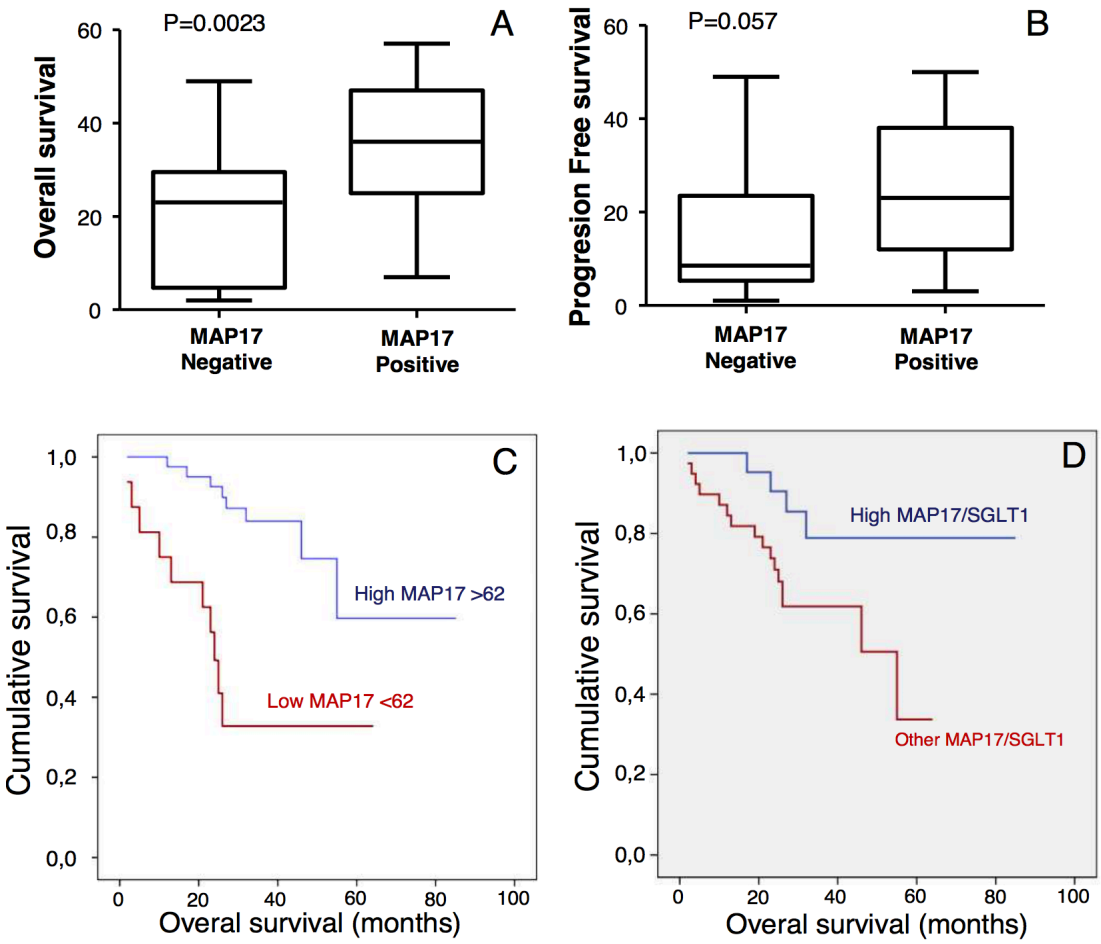


Figure 14. *MAP17 alone or in combination with SGLT1 are good independent markers for patient survival. A) Correlation of MAP17expression measured as a dichotomous variable, MAP17 high rates (>62) with overall survival. B) Correlation of MAP17 expression measured as a dichotomous variable, MAP17 high rates (>62) with laryngoesophageal dysfunction-free survival. C) A Kaplan-Meier curve is shown indicating that MAP17 could be a good prognostic marker for overall survival in laryngeal tumour patients treated with radiotherapy plus bio/chemotherapy. D) A Kaplan-Meier curve shown indicates that combined high levels of MAP17 and SGLT1 levels are a good prognostic marker for survival in laryngeal tumour patients treated with radiotherapy plus adjuvant chemotherapy.*

4.1.4. Tumour cells overexpressing MAP17 are more sensitive to radiation

To explore whether MAP17 may be causal in this response, we expressed MAP17 cDNA in Hela cells and subjected these cells and their parental expressing only empty vector, to different doses of radiotherapy. Our data showed that Hela cells expressing MAP17 (Figure 15A) were more sensitive to radiation than parental cells without MAP17 (Figure 15B), therefore confirming the causal role of MAP17 in the sensitivity to radiation. Finally, to test our initial hypothesis of the relevance of ROS in the MAP17-enhanced radiosensitivity of Hela cells, we treated the cells with

antioxidants GSH and NAC, and subjected these cells to different radiation doses. We observed that both antioxidant treatments reduced the sensitivity of MAP17-expressing Hela cells to a range similar to parental cells, which remains mostly unaltered (Figure 15C and D). These data confirm the relevance of the oxidative status of the tumours in the response to radiation.

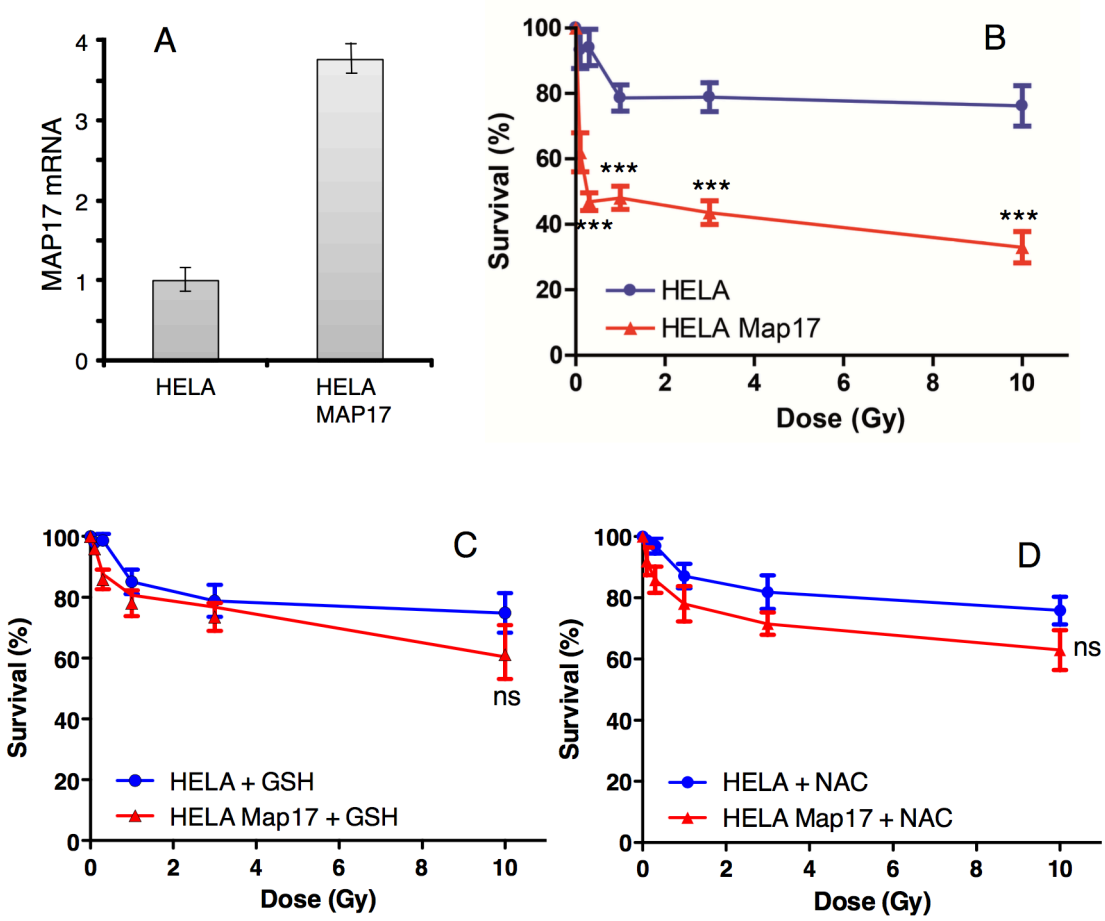


Figure 15. *MAP17 overexpression in Hela cells induces sensitivity to radiotherapy. A) Hela cancer cells expressing ectopic MAP17 cDNA were selected and analysed for MAP17 mRNA expression by quantitative RT-PCR. B) Hela cells expressing ectopically MAP17 cDNA (Hela-Map17) and parental cells expressing only empty vector (Hela) were seeded at equal concentration and subjected to different radiation doses as indicated, in triplicate samples. 48 hrs after treatment the percentage of survival cells was measured in each case and plotted in the graph. The experiment was performed three independent times in triplicate. C and D) Hela cells expressing ectopically MAP17 cDNA (Hela-Map17) and parental cells expressing only empty vector (Hela) were seeded at equal concentration and subjected to pre-treatment with 10 mM GSH (C) or 10 mM NAC (D) during 18 hrs, then treated with different radiation doses as indicated, in triplicate samples. 48 hrs after treatment the percentage of survival cells was measured in each case and plotted in the graph.*

In summary, our analysis in laryngeal cancer showed a significant relationship between high MAP17 protein expression and increased OS, suggesting that MAP17 expression is an independent biomarker for survival. In fact, high MAP17 levels demonstrated better OS than low levels (67 months vs. 31.7 months, IC 95%; $p<0.001$). High MAP17 showed better LRC and LDS as well. In addition, the associated high levels of MAP17 and SGLT showed improved OS, better than MAP17 alone. Finally, proof of principle experiments in vitro demonstrated that antioxidant treatments reduced the sensitivity of MAP17- expressing Hela cells to a range similar to parental cells, confirming the relevance of the oxidative status of the tumours in the response to radiation.

4.2. PHOSPHORYLATION OF GH2AX AS A NOVEL PROGNOSTIC BIOMARKER FOR LARYNGOESOPHAGEAL DYSFUNCTION-FREE SURVIVAL

γH2AX is a component of the histone octamer in nucleosomes that its phosphorylated (pH2AX) by ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR) and DNA-PK. PH2AX is involved in recruiting DNA repair proteins in response to the presence of DNA DSB. Therefore, it´s increasing is related to DNA damage and has been studied as a potential biomarker. Our goal was to determine whether pH2AX by itself or in combination with other molecular and clinical findings could be a prognosis biomarker for our cohort of laryngeal cancer.

4.2.1. Clinical cohort description

At the time of the analysis, 20 (32%) deaths and 29 (46%) recurrences had occurred with a median follow-up of 29m. Locoregional relapse occurred in 19 (30%) patients, 7 (11%) presented locoregional plus distant metastases, and 3 (4.8%) only distant metastases; of them, 14 (48.3%) were candidates for salvage surgery. Laryngoesophageal dysfunction (LD) occurred in 51% of the total; main reasons for LD were tumoral local recurrence (75%) followed by the need of a tracheostomy of feeding tube (15.6%). Mean OS was 58 m (47.7-68 m, CI 95%), LDS 46 m (36-55.5m, CI 95%), and LRC 54.6 m (44-65 m, CI 95%), with a 2-year LRC of 63%. The 2-year cumulative proportion of patients with larynx preservation and OS were 57% and 80% respectively. Lymph node involvement was associated with worse OS (N0: 64.2m vs N1/2: 26.8 m, $p<0.01$) but not with LDS (46.8 vs 24.6, $p=0.6$) (Figure 16B and A). Tumour local extension impacted negatively on both OS and LDS (OS non-T4 60.7 m vs T4 22.5 m; LDS non-T4 48.5 m vs T4 7.3m, both $p=.001$) (Figures 16D and C). Furthermore, patients who required pretreatment tracheotomy (PT) had worse OS (37.2 m vs 61.8 m, $p=0.051$) and LDS (19.6 m vs 55.4m, $p=0.001$) (Figures 16F and E).

4.2.2. Cisplatin and radiotherapy as prognostic markers in larynx cancer

As per the RTOG 0129 phase III clinical trial results [137], patients were classified by the dose of cisplatin received during the radiation treatment. In total, 52.4% reached the optimal dose of cisplatin during radiotherapy, whereas 17.5% could not reach the cisplatin optimal dose, 12.7% did not receive any radiosensitizer, and 14.3% were treated with other radiosensitizers. 3.1% were unknown. Cisplatin optimal dose (≥200 mg/m2) was associated with better survival, although this was statistically significant just for LDS (OS: 67 m vs 39 m, $p=0.073$; LDS: 56m vs 24 m, $p=0.017$; LRC: 60.4 m vs 29 m, $p=0.12$) (Figures 17B and A). Receiving an optimal dose of cisplatin

had better LDS (p=0.023) than lesser doses (HR=0.24), other radiosensitizers (HR=0.32), and no concurrent radiosensitizers (HR=0.65). However, this benefit was not observed for OS probably because the analysis did not take into account patients that needed salvage laryngectomy and did not preserve the organ.

Total dose of radiation delivered was 70 Gy, as per standard local guidelines. Patients that completed radiotherapy within 8 or 9 weeks were compared to those that suffered interruptions or delays but no differences were found in terms of OS or LDS between groups (Figure 18).

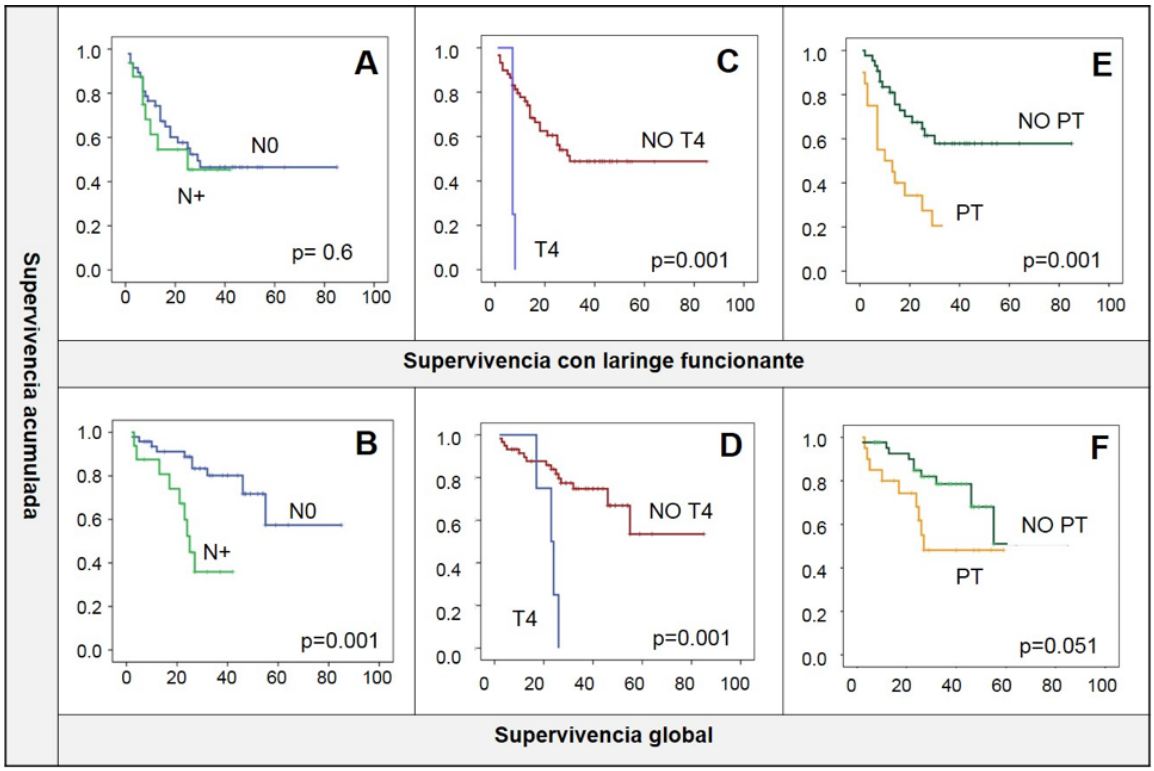


Figure 16. *A. and B. N0 compared with N positive LDS/OS. OS was better in the patients who had no lymph node involvement. C. and D. T4 local tumour extension shows worse OS and LDS than the rest of patients. E. and F. worse LDS and OS is observed in patients who required pretreatment tracheotomy (PT).*

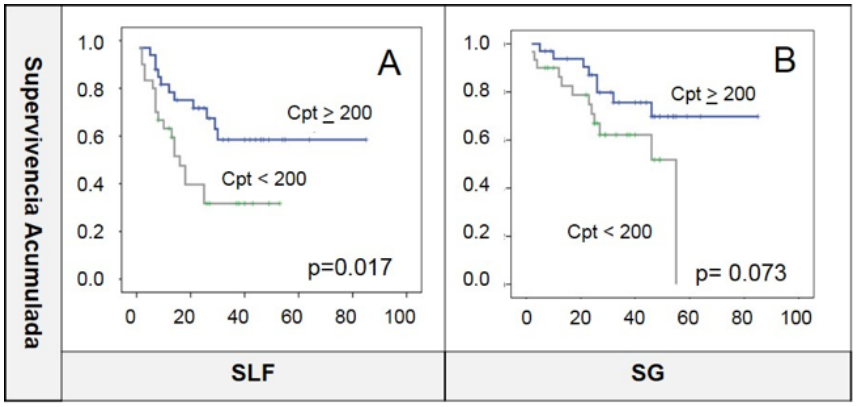


Figure 17. *A. and B. Cisplatin (Cpt) optimal dose (≥200 mg/m2) showed significant LDS bene t that was not maintained for OS.*

4.2.3. pH2AX in larynx tumour samples

Only 53 samples were analysed for pH2AX expression either due to technical problems or because they did not contain any tumour cellularity. Positive pH2AX expression, considered as any percentage of tumoral nuclei with positive staining, was shown in 46 (86.8%) samples with a range of 1 to 70 and median expression of 10 (figure 19A). In order to distinguish a cut-off point for pH2AX levels a ROC curve was performed and punctuation of 5.25 score chosen. Levels of pH2AX were equally distributed among tumour grades (Figure 19B) suggesting independence from this clinical feature, as the Chi-square test showed no differences between groups (p=0.8).

When measured as a continuous variable, pH2AX had a significant positive influence with better LDS outcomes (HR 0.95, p=0.02), although this was not significant for OS. As a dichotomous variable, a trend towards better OS, LDS and LRC was observed but just LDS was statistically significant in the multivariate analysis (HR 0.26, p=0.02) (Table 2) (Figures 19C, D and E).

We also studied the potential correlation between pH2AX and clinical findings such as tumour localization, tumoral stage, smoking habit, alcohol consumption and acute toxicity development with no statistically significant association. These results suggest pH2AX to be an independent prognostic factor, as it remains significant after controlling for these variables.

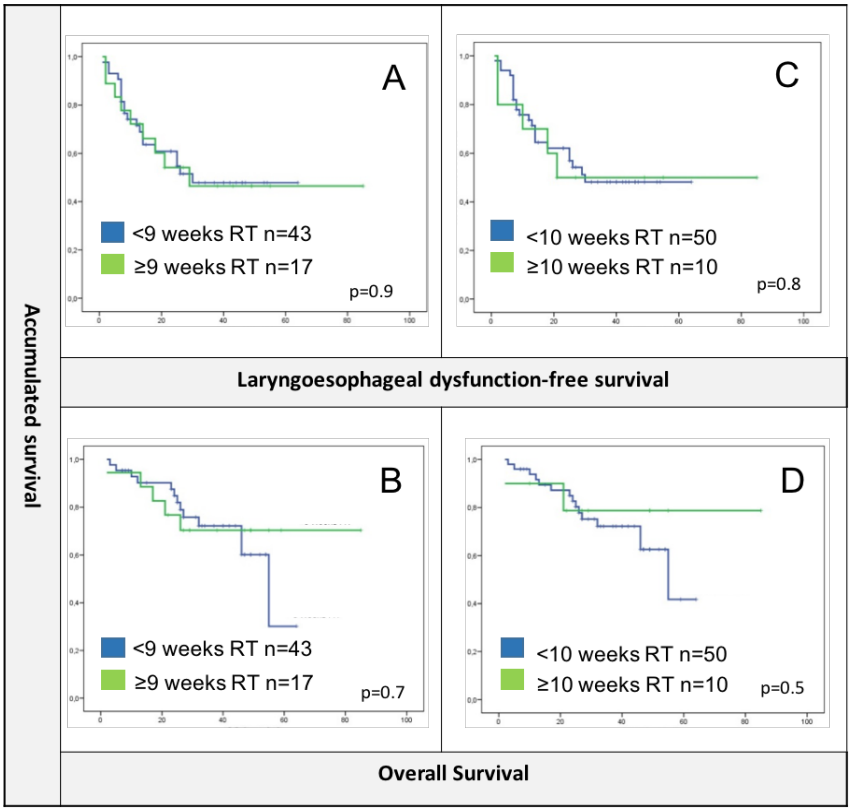


Figure 18. *A. and B. No differences were found for radiotherapy delivered within less than 9 weeks or ≥9 weeks in terms of LDS or OS. C and D. Same results for a cut-off of 10 weeks.*

LDS				OS			
	HR	***p*-value**	**CI**		**HR**	***p*-value**	**CI**
PT	1.41	0.59	0.39-5.08	**PT**	0.96	0.98	0.17-5.58
Non-primary T4 extension	0.09	0.00	0.02-0.49	**N negative**	0.65	0.03	0.01-0-74
Cisplatin >200mg/m²	0.09	0.00	0.02-0.43	**Cisplatin >200mg/m²**	0.33	0.29	0.04-2.56
High-pH2AX	0.26	0.02	0.09-0.78	**High-pH2AX**	0.57	0.46	0.13-2.55
KI 67	1.03	0.15	0.99-1.06	**KI 67**	1.06	0.038	1.00-1.12
MAP17	1.01	0.63	0.99-1.02	**MAP17**	0.98	0.05	0.95-1.00
P53	0.99	0.37	0.56-1.02	**P53**	1.03	0.12	0.99-1.07
pERK	0.99	0.35	0.97-1.01	**pERK**	1.01	0.65	0.98-1.04
pAKTP	0.99	0.41	0.98-1.01	**pAKT**	0.98	0.16	0.96-1.01

Table 8. *LDS and OS multivariate analysis. PT: pretreatment tracheotomy. N: pathological lymph nodes.*

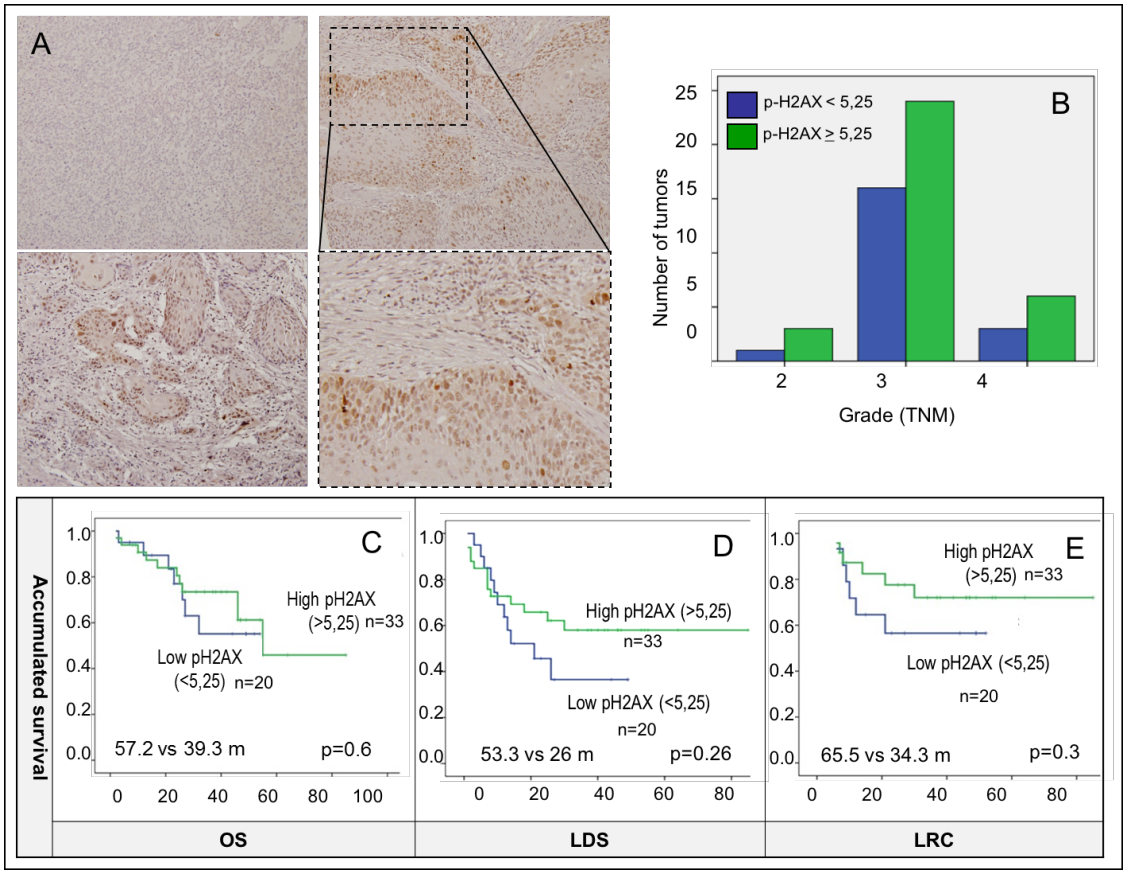


Figure 19. *A. Positive pH2AX expression, considered as nuclei staining was shown in 46 (86.8%) samples. B. Levels of pH2AX are equally distributed among tumour stages. C., D. and E. high-pH2AX show a trend towards better OS, LDS and LRC not statistically significant in the Kaplan-Meier analysis. 5.25 as indicated by the ROC curve were used as cut-off for defining high and low expression of pH2AX for survival analysis.*

4.2.4. pH2AX relationship with cisplatin and radiotherapy

The total dose of cisplatin was not associated with pH2AX levels (p=0.4). We created a variable with two categories from pH2AX and cisplatin, in which one had a potential favourable prognosis (high-pH2AX levels, and optimal dose of cisplatin, ≥200 mg/m2), and the other unfavourable prognosis (low-pH2AX levels, and/or suboptimal dose of cisplatin <200mg/m2 or other radiosensitizers due to the low number of patients). The favourable prognosis group correlated with increased OS, LDS (OS: 72 m vs 38.6 m, p=0.03; LDS 66.9 m vs 27 m, p=0.019). LRC was not statistically significant (p=0.17) although there was a trend towards better outcomes in the good prognostic subgroup (69.9 m vs 35.1 m) (Figures 20A, B and C). Moreover, the unfavourable prognosis group correlated with worse OS (HR= 3.66, p=0.044), and LDS (HR= 3.38, p=0.028). LRC has a not statistically significant HR (HR=2.4, p=0.188). We also tried to

stablish whether high-pH2AX and no radiotherapy delays could impact on survival but no differences were found for both OS and LDS.

4.2.5. Correlation of pH2AX with p53 and KI67

We also analysed other markers for proliferation such as KI67 or the activated form of ERK (phosphorylated ERK, ERK-p), and apoptosis such as mutant (m) p53 or activated AKT (phosphorylated AKT, AKT-p). Our cohort showed a percentage of positive samples for ERK-p or AKT-p, but these groups did not show correlation with pH2AX expression (data not shown). KI67 in combination with pH2AX was not significant in any combination (data not shown), being pH2AX also independent of the proliferative capability of the tumour. Our results showed no correlation between p53 and pH2AX although there was a relation towards increased pH2AX with negative P53 (<5% positive nuclei) that was not statistically significant (p=0.33).

However, in our cohort p53 samples positive (measured as >5% positive nuclei) (Figure 21A, + p53) correlated with worse OS (- p53=50 vs + p53=35.6 m, p=0.05) (Figure 21B) consistent with previous literature [151].

P53 and pH2AX were combined into a new variable with the following categories: potential good prognosis phenotype (negative p53 and high-pH2AX) and unfavourable prognosis phenotype (positive p53 and low-pH2AX). Although there was an apparent relation towards better outcomes in the good prognosis phenotype, this was not significant for both OS and LDS (OS: 48.6 m vs 39 m, p=0.39; LDS: 38.8 m vs 24.4 m, p=0.068) (Figures 21C and D).

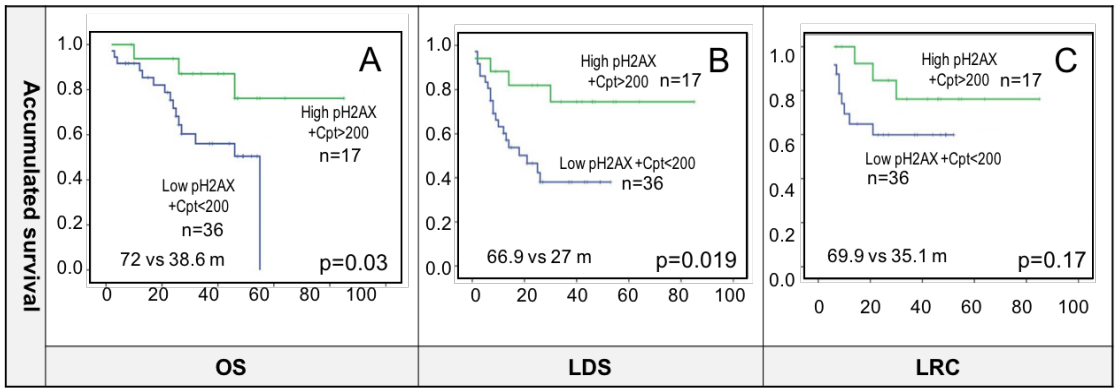


***Figure 20**. A., B. and C. pH2AX and dose of concomitant cisplatin were combined in a new variable where high-pH2AX and cisplatin (Cpt) ≥200 mg/m2 was considered as good prognosis phenotype category. The results show improved OS and LDS in this subgroup, and a trend towards better LRC. 5.25 as indicated by the ROC curve were used as cut-off for defining high and low expression of pH2AX for survival analysis.*

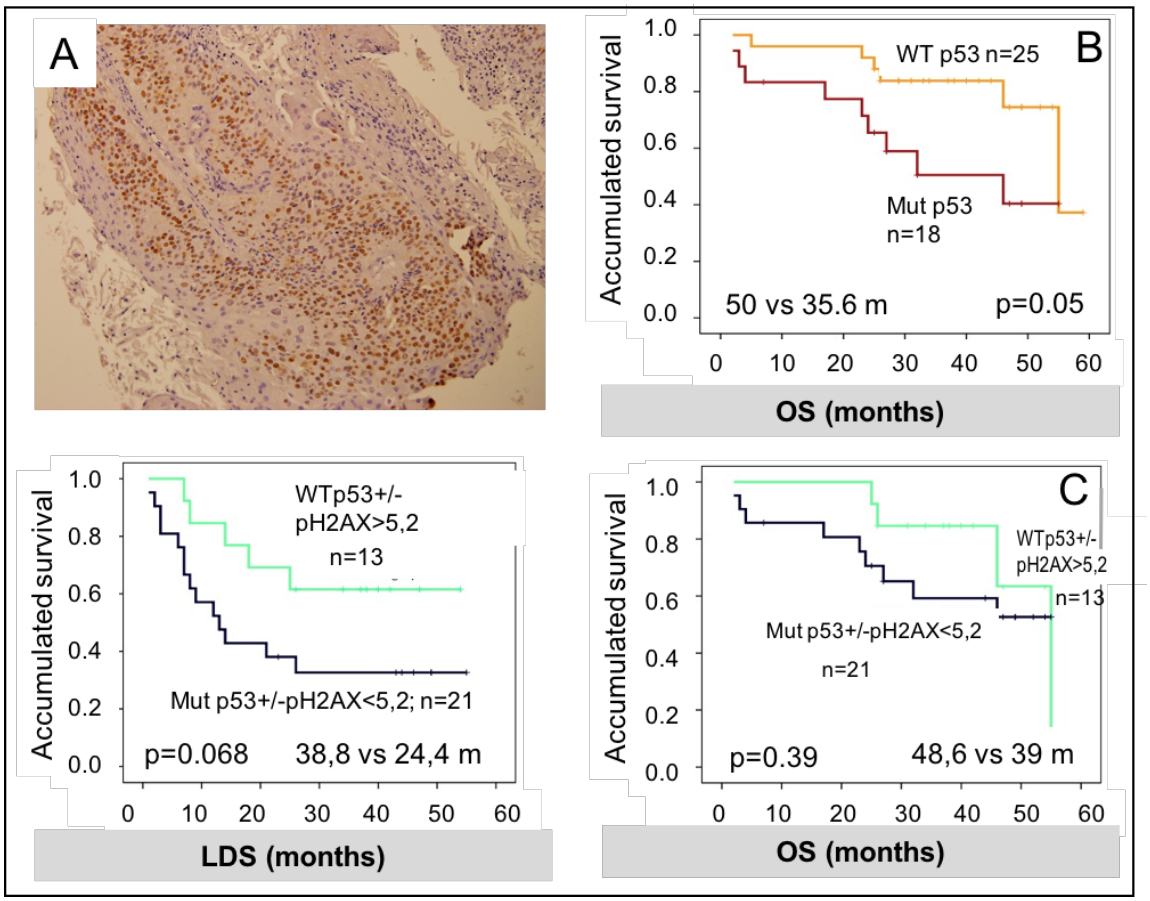


Figure 21. *A. P53 was measured as >5% positive nuclei, as shown in the picture. B. positive P53 (+ P53) correlates with worse OS in our cohort. C. and D. results of the combination of P53 and pH2AX in a new variable. Although there was a trend towards better outcomes in the good prognosis phenotype which included negative P53 (-P53) and high-pH2AX, this was not statistically significant for OS and LDS.*

4.2.6. Correlation of pH2AX and MAP17

As discussed before, MAP17 increases endogenous ROS, which is a well-known mediator of DNA damage. Therefore, we measured whether pH2AX correlated with MAP17 expression and if the combination of both markers could strength the predictability of responses. We found that patients with high levels of MAP17 and subject to optimal doses of cisplatin had better LDS (58.6 m vs 32.6 m, p=0.053) and OS (76.2 m vs 40.9 m, p=0.005) than patients with low MAP17 or not subject to optimal doses of cisplatin (Figures 22A and 7B). Furthermore, patients with high levels of MAP17 and high-pH2AX, denoting higher structural DNA-damage, conformed the group of better prognosis after therapy (Figures 22C and 7D).

Moreover, patients with high-MAP17, high-pH2AX and optimal dose of cisplatin had better OS and LDS than the rest of the population (Figures 22E and F), and also when compared with patients having a poor prognosis phenotype (low-MAP17, low-pH2AX and suboptimal cisplatin dose) (Figures 22G and H).

In summary, these results show that pH2AX has a prognostic role in patients with laryngeal cancer. pH2AX was related to LDS (High- pH2AX HR 0.26, p = 0.02) in a cohort of 53 patients with larynx cancer. When analysed together pH2AX expression and dose of cisplatin received during radical treatment, there is a significant correlation with survival (high-pH2AX and optimal dose of cisplatin 72 vs 38.6 m, p = 0.03) and LDS (high-pH2AX and optimal dose of cisplatin 66.9 vs 27 m, p = 0.019). Also, patients with high-MAP17 and high-pH2AX showed to have better OS and LDS. Our data also show the importance of performing optimal cisplatin treatment for tumour response. However, the fact that unexpected radiotherapy delays and interruptions did not affect survival in our cohort could be explained to dose compensations. Radiobiological-based calculations were performed in those patients in order to achieve an equivalent biological effectiveness by adding some more fractions to the overall treatment. Our data suggest that inherent DDR pathway activation (measured by the end-point of phosphorylation of H2AX) is a valuable prognostic marker in patients with laryngeal carcinoma who received organ preservation approaches.

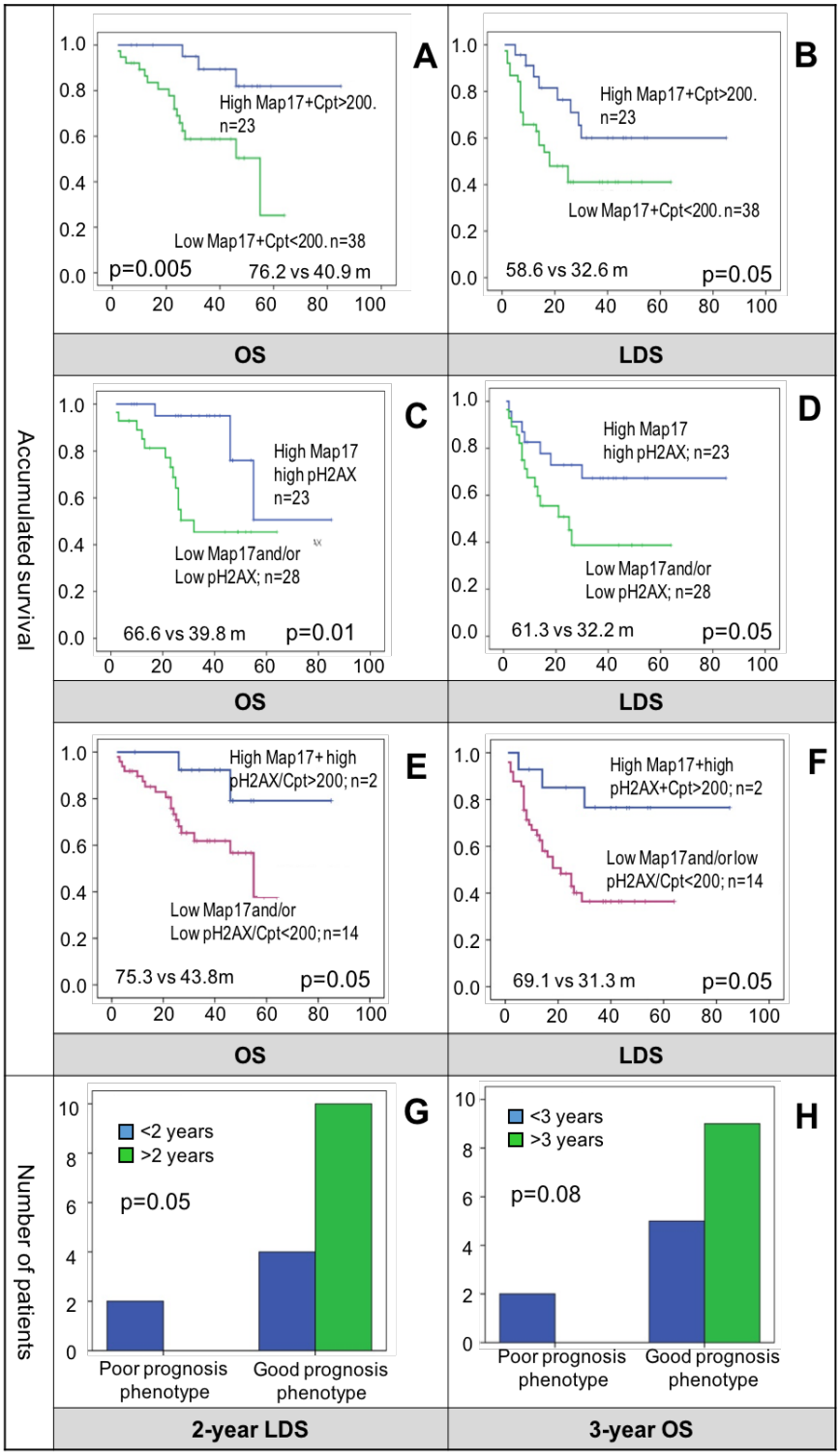


Figure 22. *A. and B. the combination of high-MAP17 and optimal doses of cisplatin (Cpt) showed better OS and LDS. C. and D. patients with high-MAP17 and high-pH2AX with higher structural DNA-damage showed to have better OS and LDS. E. and F. survival for patients with high-pH2AX, high-MAP17 and optimal dose of cisplatin was statistically better. G. and H. the subgroup of patients with high-pH2AX, high-MAP17 and cisplatin optimal dose patients was compared to the patients that had low-pH2AX, low-MAP17 and did not complete cisplatin. Although limited in numbers, none of the patients with poor prognosis phenotype reached more than 2-years LDS or more than 3-years OS.*

In this work novel biomarkers for patients diagnosed of laryngeal cancer who were candidates for organ preservation treatments have been identified. In general, accepted organ sparing approaches include radiotherapy, bio or chemotherapy with concomitant radiotherapy and induction chemotherapy followed by radiotherapy with or without bio/chemotherapy. To date, there are no biomarkers validated that could predict survival or response with these preservation approaches, and a number of patients have significant late toxicities or finally relapse. In those cases, salvage surgery can sometimes be performed. Identifying predictive biomarkers in this setting could avoid unnecessary toxicities and could potentially improve survival because of the better selection of patients.

Pathological, clinical and therapeutic features of our population were studied in order to determine whether they could be related to survival. In this regard, patients with T4 primary tumour and those who needed a pre-treatment tracheotomy had worse LDS and seemed not to benefit of preservation treatments. Moreover, receiving optimal platinum total dose can determine LDS, as patients unable to complete treatment have a worse prognosis.

A panel of different markers were studied in our population; proliferation markers such as Ki67 or p-ERK, apoptosis such as p53 and p-AKT. However, DNA damage response biomarkers such as MAP17, SGLT and pH2AX were the ones implicated with survival in our cohort.

MAP17 is a membrane-associated protein known to increase endogenous ROS through SGLT1 in cancer cells [152,153]. ROS are well known mediators of DNA damage. In a previous study, expression of both MAP17 and SGLT1 was associated with survival in a cohort of patients with squamous cervical cancer treated with radiotherapy and cisplatin [107], thus having some similarities to our population. Those results suggested that patients expressing MAP17 and SGLT1 had better response to treatments that boost oxidative stress. In a more recent study, high MAP17 levels correlated with a poor prognosis and a higher grade of sarcoma. In this case, it was a heterogeneous group of different sarcoma histologies, most of them with metastatic disease. As patients were candidates for systemic therapies and not for radiotherapy, oxidative stress may not be implicated in this population [154]. Our analysis in laryngeal cancer showed that MAP17 increased not only OS but also LRC and LDS in laryngeal cancer. On the other hand, SGLT-1 expression was significantly related to MAP17. However, just a portion of tumour samples was positive (40%) and therefore survival could not be related to SGLT1 by itself. Nevertheless, the combination of high MAP17 and SGLT improved OS better than MAP17 alone.

γH2AX phosphorylation has been studied as prognostic biomarker in early operable non-small cell lung cancer (NSCLC) and endometrial carcinomas. In the NSCLC study, low levels of pH2AX correlated with better survival outcomes. The combination of wild type p53 and low-pH2AX phenotype showed also better survival. In the endometrial trial, pH2AX positively

correlated with p53 levels although the relation with survival could not be proved. However, patients in both studies were treated with surgery and not with radiotherapy [155, 156]. Tumour cells from clinical specimens show constitutive activation of DNA damage signalling as demonstrated by the presence of gH2AX phosphorylation and other DDR signalling proteins [157,158, 159]. This DDR activation was found to peak at early stage tumours, persisting further among malignant tumours mostly by inactivating p53 gatekeeper [159]. It has been proposed that the DDR-network may serve as an inducible barrier to control the initial steps of tumour development by inducing p53-dependent senescence or apoptosis [157,158, 159]. Further ongoing chronic DDR activation favours the outgrowth of malignant clones with genetic or epigenetic defects in DNA-repair mechanism such as those involved in the DDR pathway [159]. Our samples, from already malignant tumours (stages II-IV), in which only a subset of them showed mutant p53, correlated with worse onset of the disease. It is likely DNA-damage defects inducing DDR activation have been carried through the malignant process and it is possible that other proteins are mutated in the process avoiding the requirement for p53 inactivation.

pH2AH is a broad DNA damage marker that appears under different physiological conditions. Senescent cells display molecular characteristics of DNA damage [160,161,162]. These markers include nuclear foci of phosphorylated histone H2AX, the localization at double-strand break sites of DNA-repair and DNA-damage checkpoint factors, such as 53BP1, MDC1 and NBS1 [163, 164, 165]. Senescent cells also contain activated forms of the DNA-damage checkpoint kinases Chk1 and Chk2. During replicative senescence, markers of a DNA damage response localize at telomeres [164, 166], indicating that the DNA damage response is triggered by telomere shortening [167]. Similarly, the redox potential also results in DNA damage and senescence (168). Very interestingly, oncogene-induced senescence has been found to induce DNA-damage due to an excess of replication forks. This oncogenic-induced hyper-replication signal, or replication stress, is associated with persistent DNA-damage [169, 170] inducing senescence [171]. Therefore, not only senescence is viewed as a response to DNA-damage, but DNA-damage as a marker of senescence. In that sense, high pH2AX appeared in early stage tumors and is a marker of good prognosis [172,173]. However, in our cohort, pH2AX levels are increased in advanced stages of tumors, and contrarily to this hypothesis, are a marker of bad prognosis, indicating that our pH2AX observations are not due to cellular senescence, neither by continuous proliferation nor by replication stress.

In that line, phosphorylation of H2AX is not always a marker of DNA damage. It also can be a marker of activated mTOR, eliciting replicative stress and a pseudo DNA-damage in senescent cells [170, 174-177]. The dynamics of senescence exhibit 2 different steps: cell cycle arrest and further acquisition of senescence features, which includes permanent arrest, termed geroconversion [170, 178-180]. If geroconversion is not activated, cells are only transiently arrested with the possibility of

resuming growth once the proliferation constraints have been eliminated [163, 180]. It has also been shown that if mTOR is activated under conditions of proliferative arrest, then arrest becomes permanent and the cell undergoes senescence [178, 179, 181]. Under these conditions of cell cycle arrest and mTOR activation, the phosphorylation of H2AX is launched, becoming a marker of cellular senescence [165, 171, 174, 181]. In fact, rapamycin treatment, which inhibits mTOR, can divert senescence into quiescence, allowing the cell to resume growth once conditions are more favourable [182-185]. Since mTOR is the master regulator of protein synthesis [186], it has been proposed that this contribution is due to the function of mTOR as a sensor of cellular nutrients and energy status as well as growth factor signals [187, 188]. However, it has also been reported that mTOR activation in the context of growth arrest is perceived by the cells as an unwanted oncogenic signal, activating the replicative stress and pseudo DNA-damage signalling [165, 171, 174, 181]. In any case, high levels of pH2AX as marker of cellular senescence should be associated to better prognosis, and to some extent to early stage tumours. However, it will be of interest to correlate the levels of pH2AH with those of mTOR activation in laryngeal tumours to provide a more accurate hypothesis of the pH2AH inducers.

Our data show that high levels of pH2AX correlate with better prognosis after treatment with DNA-damage agents such as cisplatin and radiotherapy, especially if cisplatin is given at optimal doses. These data are suggestive of a collaboration of DDR pathway activation, perhaps as an indicator of low DNA-repair ability and DNA-damaging agents in tumour therapy. The fact that doses of cisplatin are important for survival seems to confirm this hypothesis. In line with this, wt-P53 with high levels of pH2AX conforms a subgroup of good prognosis suggesting that P53 activity is essential to drive physiological response to apoptosis (or senescence) of DNA-damage agents in tumours with DDR activated.

These data are opposite to the found in early operable non-small cell lung cancer (NSCLC). In this study, low levels of pH2AX correlated with better survival outcomes. The combination of wild type p53 and low-phosphorylated ãH2AX phenotype showed also better survival. However, NSCLC patients were treated with surgery and not with radiotherapy [155]. This lack of treatment with radiotherapy could be the cause of the different behaviour respect the pH2AX. Radiotherapy increases oxidative stress and reactive oxygen species that in combination with pre-existing DNA damage can increase cell damage above threshold inducing increased tumour efficacy. Our data support this hypothesis since combination with another ROS-inducing agent such as cisplatin is essential to gain better survival in these patients. Furthermore, the combination of MAP17 also supports the essential role of radiotherapy in this response.

Therefore, this work suggests that high levels of MAP17 induced ROS that in turn increases DNA-damage and DDR signalling. Upon further DNA-damage and further increase in ROS molecules induced by cisplatin and RT treatment, tumours with higher oxidative stress (higher

MAP17, higher ROS denoted by higher pH2AX), are more suitable to undergo apoptosis in the presence of P53 activity. Our data seems to confirm that MAP17 and pH2AX are markers of structural DNA-damage in the laryngeal tumours that may become novel and valuable prognostic biomarkers for laryngeal carcinoma. Further prospective and controlled studies are needed in order to confirm these results and validate MAP17 and pH2AX as biomarkers for clinical use in larynx cancer.

CONCLUSIONS

- In our cohort, T4 primary tumour extension and patients who need a pre-treatment tracheotomy have worse LDS and do not to benefit of preservation treatments. Moreover, receiving an optimal platinum dose determined LDS.

- Our analysis in laryngeal cancer showed a significant relationship between high MAP17 protein expression and increased OS, suggesting that MAP17 expression is an independent biomarker for survival.

- High MAP17 levels demonstrated better OS than low levels (67 months vs. 31.7 months, IC 95%; $p<0.001$). High MAP17 showed better LRC and LDS as well.

- In addition, the associated high levels of MAP17 and SGLT showed improved OS, better than MAP17 alone.

- Proof of concept experiments in vitro demonstrated that MAP17- expressing Hela cells are more sensitive to radiotherapy treatments *in vitro*.

- Furthermore, antioxidant treatments reduced the sensitivity of MAP17- expressing Hela cells to a range similar to parental cells, confirming the relevance of the oxidative status of the tumours in the response to radiation.

- pH2AX has a prognostic role in patients with laryngeal cancer. pH2AX was related to LDS (High- pH2AX HR 0.26, $p = 0.02$) in a cohort of 53 patients with larynx cancer.

- When analysed together high-pH2AX expression and optimal dose of cisplatin received during radical treatment, there is a significant correlation with survival (72 vs 38.6 m, $p= 0.03$) and LDS (66.9 vs 27 m, $p = 0.019$).

- Also, patients with high-MAP17 and high-pH2AX showed to have better OS and LDS.

- Our data suggest that inherent DDR pathway activation (measured by the end-point of phosphorylation of H2AX) is a valuable prognostic marker in patients with laryngeal carcinoma who received organ preservation approaches.

CONCLUSIONES

- En esta cohorte, los pacientes con afectación de tumor T4 y aquellos que requirieron traqueotomía previa al tratamiento obtuvieron peor SLF y no se beneficiaron de las terapias de preservación de órgano. Además, recibir una dosis de platino óptima determinaron la SLP.

- En este estudio se demuestra la relación entre el aumento de la expresión proteica de MAP17 y el aumento de SG, sugiriendo que MAP17 es un biomarcador independiente de supervivencia.

- Niveles altos de MAP17 se asocian a un aumento significativo de la SG en comparación con niveles bajos (67 m vs. 31.7 m, IC 95%; $p<0.001$). Alto-MAP17 también se correlaciona con CLR y SLF.

- Además, el análisis conjunto de alto-MAP17 y SGLT aumentan la SG, mejor que el análisis independiente de MAP17.

- La prueba de concepto demuestra que la expresión de MAP17 en células Hela confiere sensibilidad a la radioterapia *in vitro*.

- De hecho, la adición de tratamientos antioxidantes redujeron la sensibilidad de la línea celular Hela con alta expresión de MAP17 hasta un rango similar a las células madre, confirmando la relevancia del estado oxidativo de los tumores para su respuesta a la radioterapia.

- pH2AX es un factor pronóstico asociado a SLF (alto-pH2AX HR 0.26, $p=0.02$) en una cohorte de 53 pacientes con cáncer de laringe.

- El análisis conjunto de alto-pH2AX y dosis óptima de cisplatino recibido ha demostrado de forma significativa aumentar la SG (72 vs 38.6 m, $p = 0.03$) y la SLF (66.9 vs 27 m, $p= 0.019$).

- Además, los pacientes con alto-MAP17 y alto-pH2AX obtuvieron mejor SG y SLF.

- Los datos de este estudio sugieren que la activación inherente de los mecanismos de RDA, medido por la fosforilación de H2AX es un factor pronóstico en pacientes con cáncer de laringe que han recibido terapias de preservación de órgano.

REFERENCES

1. Ferlay J, Soerjomataram I, Ervik M, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase.

Accessed: http://globocan.iarc.fr (link is external), accessed December 2013.

2. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, et al. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries in 2012. European Journal of Cancer (2013) 49, 1374-1403.

3. Fitzmaurice C, Dicker D, Pain A, et al. The global burden of cancer 2013. JAMA Oncol. 2015 July 1; 1(4): 505-527.

4. SEER Cancer Statistics Factsheets: Larynx Cancer. National Cancer Institute.

Accessed: http://seer.cancer.gov/statfacts/html/laryn.html

5. Red Española de registros del cancer.

Accesed: http://redecan.org/es/download_file.cfm?file=257&area=196

6. Spanish Society of Medical Oncology annual report, "Las cifras del cáncer en España 2016". Accesed: http://www.seom.org/seomcms/images/stories/recursos/LAS_CIFRAS_DEL_CANCER_EN_ESP_2016.pdf

7. Mehta V, Shi Z, Mills GM, et al. Effect of Payer Status on Relative Survival of Patients with Laryngeal Cancer. Anticancer Res. 2016 Jan;36(1):327-33

8. Cancer Research UK Cancer Survival Group at the London School of Hygiene and Tropical Medicine.

Accesed: http://www.lshtm.ac.uk/eph/ncde/cancersurvival/http://www.lshtm.ac.uk/eph/ncde/cancersurvival/

9. Bobdey S, Jain A, Balasubramanium G. Epidemiological review of laryngeal cancer: An Indian perspective. Indian J Med Paediatr Oncol. 2015 Jul-Sep;36(3):154-60.

10. Medscape Larynx anatomy.

Accessed: http://emedicine.medscape.com/article/1949369-overview

11. Standring S. Gray´s Anatomy, the anatomical basis of clinical practice. 41st Edition, 2016. ISBN: 978-0-7020-5230-9

12. Merati AL, Bielamowicz SA. Textbook of Laryngology. San Diego: Plural Publishing Inc; 2006.

13. Ross MH, Pawlina W. Histology: A Text and Atlas with Correlated Cell and Molecular Biology. 6th. Philadelphia: Lippincott Williams & Williams; 2010.

14. Lalwani AK. Current Diagnosis & Treatment Otolaryngology: Head and Neck Surgery. 3rd ed. New York: McGraw-Hill Medical; 2011.

15. Flint PW, Haughey BH, Lund VJ, et al. Cummings Otolaryngology – Head and Neck Surgery. 5th. Philadelphia: Mosby; 2010. 1:

16. Netter F. Atlas de Anatomía Humana, 2ª edición. 1999. 0-914168-86-X.

17. Stiblar-Martincic D. Histology of laryngeal mucosa. Acta Otolaryngol Suppl. 1997;527:138-41.

18. Orvidas LJ, Olsen KD, Lewis JE, Suman VJ. Verrucous carcinoma of the larynx: a review of 53 patients. Head Neck. 1998 May;20(3):197-203.

19. Shahid-Iqbal M, Paleri V, Brown J. Spindle cell carcinoma of the head and neck region: treatment and outcomes of 15 patients. Ecancermedicalscience. 2015; 9: 594.

20. Mastronikolis, NS, Papadas, TA, Goumas, P. Head, neck: Laryngeal tumors: an overview. Atlas Genet Cytogenet Oncol Haematol. 2009;13(11):888-893.

21. DeVita VT, Lawrence TS, Rosenberg SA. Cancer, principles & practice of Oncology. 8th Edition, 2008. ISBN: 978-0-78177207-5.

22. Pillsbury HR, Kirchner JA. Clinical vs histopathological staging in laryngeal cancer. Arch Otolaryngol 1979; 105:157.

23. Lindberg RD. Distribution of cervical lymph node metastases from SCC of the upper respiratory and digestive tracts. Cancer 1972;29:1556

24. Mendelhall WM, Amdur RJ, Morris CG. T1-T2N0 SCCof the glottic larynx treated with radiation therapy. J CLin Oncol 2001;19:4029

25. Olsen KD, DeSanto LW, Pearson BW. Positive Delphian lymph node: clinical significance in laryngeal cancer. Laryngoscope 1987;97:1033.

26. Lederman M. The place of radiotherapy in the treatment of cancer of the larynx. Ann Radiol 1961;4:443.

27. Buffalo SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A. Cancer Staging Manual. Seventh Edition. ISBN: 978-0-387-88440-0.

28. American Cancer Society.

Accessed at: https://www.cancer.org/cancer/laryngeal-and-hypopharyngeal-cancer/detection-diagnosis-staging/survival-rates.html

29. Maier, H, Weidauer, H. Alcohol drinking and tobacco smoking are the chief risk factors for ENT tumors: Increased incidence of mouth cavity, pharyngeal, and laryngeal carcinomas. Fortshr. Med. 1995, 113, 157-160.

30. Gandini S, Botteri E, Iodice S. Tobacco smoking and cancer: a meta-analysis. Int J Cancer. 2008 Jan 1;122(1):155-64.

31. Berthiller J, Straif K, Agudo A. Low frequency of cigarette smoking and the risk of head and neck cancer in the INHANCE consortium pooled analysis. Int J Epidemiol. 2015 Jul 30.

32. Wyss A, Hashibe M, Chuang SC. Cigarette, cigar, and pipe smoking and the risk of head and neck cancers: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. Am J Epidemiol. 2013 Sep 1;178(5):679-90.

33. Cinciripini EM, Gritz, ER, Tsoh, JY. Smoking cessation and cancer prevention. Psycho-Oncology (J. C. Holland, ed.). Oxford Univ. Press, New York. 1998.

34. Gritz, E. R. (1991). Smoking and smoking cessation in cancer patients. Br. J. Addict. 86, 549-554.

35. Browman GE, Wong G, Hodson I. Influence of cigarette smoking on the efficacy of radiation therapy in head and neck cancer. N. Engl. J. Med. 1993, 328, 59-63.

36. Stevens MH, Gardner JW, Parkin JL. Head and neck cancer survival and lifestyle change. Arch. Otolaryngol. 1983, 109, 746-749.

37. Day GL, Blot WJ, Shore RE. Second cancers following oral and pharyngeal cancers: role of tobacco and alcohol. JNCI 1994, 86, 131-137.

38. Hiyama,T, Sato,T, Yoshino,K. Second primary cancer following laryngeal cancer with special reference to smoking habits. Japan J. Cancer Res. 1992, 83, 334-339

39. Rugg, T, Saunders M. L, Dische S. Smoking and mucosal reactions to radiotherapy. Br. J. Radiat. 1990, 63, 554-556.

40. Dresler CM, Roper C, Patterson GA. Effect of physician advice on smoking cessation in patients undergoing thoracotomy. ABS Chest 1993, 104, 18.

41. Benowitz, NL. Pharmacologic aspects of cigarette smoking and nicotine addiction. N. Engl. J. Med. 1998, 319, 1318-1330.

42. GHK Consulting, the University of Exeter in the UK and the Public Health Advocacy Institute.

Accessed at:
https://ec.europa.eu/health/sites/health/files/tobacco/docs/tobacco_liability_final_en.pdf

43. Wynder EL, Bros IJ, Day. A study of environmental factors in cancer of the larynx. Cancer 1956;9(1):86–110.

44. IARC Working Group. Alcohol drinking. IARC monographs on the evaluation of carcinogenic risks to humans, vol. 44. Lyon: IARC Press; 1988.

45. Secretan B, Straif K, Baan R. A review of human carcinogens – Part E: tobacco, areca nut, alcohol, coal smoke, and salted fish. Lancet Oncol 2009;10(11):1033–4.

46. Bagnardi V, Blangiardo M, La Vecchia C. A meta-analysis of alcohol drinking and cancer risk. Br J Cancer 2001;85(11):1700–5.

47. Islami F, Tramacero I, Rota M. Alcohol drinking and laryngeal cancer: Overall and dose–risk relation – A systematic review and meta-analysis. Oral Oncology 46 (2010) 802–810

48. Ang KK, Harris J, Wheeler R. Human papillomavirus and survival of patients with oropharyngeal cancer. The New England journal of medicine. 2010; 363:24-35.

49. Dayyani F, Etzel CJ, Liu M. Meta-analysis of the impact of human papillomavirus (HPV) on cancer risk and overall survival in head and neck squamous cell carcinomas (HNSCC). Head & neck oncology. 2010; 2:15.

50. Castellsagué X, Alemany L, Quer M. HPV Involvement in Head and Neck Cancers: Comprehensive Assessment of Biomarkers in 3680 Patients. J Natl Cancer Inst. 2016 Jan 28;108(6).

51. Bishop JA, Ma XJ, Wang H. Detection of transcriptionally active high-risk HPV in patients with head and neck squamous cell carcinoma as visualized by a novel E6/E7 mRNA in situ hybridization method. Am J Surg Pathol 36(12): 1874–1882.

52. Lewis Jr. JS, Ukpo OC, Ma XJ. Transcriptionally-active high-risk human papillomavirus is rare in oral cavity and laryngeal/hypopharyngeal squamous cell carcinomas–a tissue microarray study utilizing E6/E7 mRNA in situ hybridization. Histopathology 60(6): 982–991.

53. Chernock RD, Wang X, Gao G. Detection and significance of human papillomavirus, CDKN2A(p16) and CDKN1A(p21) expression in squamous cell carcinoma of the larynx. Mod Pathol 26(2): 223–231.

54. Peng WJ, Mi J, Jiang YH. Asbestos exposure and laryngeal cancer mortality. Laryngoscope. 2016 May;126(5):1169-74.

55. Menvielle G, Fayossé A, Radoï L. The joint effect of asbestos exposure, tobacco smoking and alcohol drinking on laryngeal cancer risk: evidence from the French population-based case-control study, ICARE. Occup Environ Med. 2016 Jan;73(1):28-33.

56. Shangina O, Brennan P, Szeszenia-Dabrowska N. Occupational exposure and laryngeal and hypopharyngeal cancer risk in central and eastern Europe. Am J Epidemiol. 2006 Aug 15;164(4):367-75.

57. Zhang D, Zhou J, Chen B. Gastroesophageal reflux and carcinoma of larynx or pharynx: a meta-analysis. Acta Otolaryngol. 2014 Oct;134(10):982-9.

58. Foulkes WD1, Brunet JS, Sieh W, Black MJ, Shenouda G, Narod SA. Familial risks of squamous cell carcinoma of the head and neck: retrospective case-control study. BMJ. 1996 Sep 21;313(7059):716-21.

59. Negri E, Boffetta P, Berthiller J. Family history of cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. Int J Cancer. 2009; 124(2): 394-401.

60. Edefonti V, Hashibe M, Parpinel M. Natural vitamin C intake and the risk of head and neck cancer: A pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. Int J Cancer. 2015 Jul 15;137(2):448-62.

61. Maasland DH, van den Brandt PA, Kremer B. Consumption of vegetables and fruits and risk of subtypes of head-neck cancer in the Netherlands Cohort Study. Int J Cancer. 2015 Mar 1;136(5):E396-409.

62. Eskiizmir G, Tanyeri Toker G, Celik O. Predictive and prognostic factors for patients with locoregionally advanced laryngeal carcinoma treated with surgical multimodality protocol. Eur Arch Otorhinolaryngol. 2017 Mar;274(3):1701-1711.

63. Wolf GT, Hong WK, Fisher SG. Induction chemotherapy plus radiation compared with surgery plus radiation in patients with advanced laryngeal cancer. The Department of Veterans Affairs Laryngeal Cancer Study Group. N Eng J Med. 1991; 324:1685-90.

64. Singh B, Bhaya M, Stern J, Roland JT, Zimbler M, Rosenfeld RM, Har-El G, Lucente FE. Validation of the Charlson comorbidity index in patients with head and neck cancer: a multi-institutional study. Laryngoscope 1997; 107(9 11 Pt1):1469-75.

65. Zhang SY, Lu ZM, Luo XN, Chen LS, Ge PJ, Song XH, Chen SH, Wu YL. Retrospective analysis of prognostic factors in 205 patients with laryngeal squamous cell carcinoma who underwent surgical treatment. PLoS One. 2013 Apr 4;8(4):e60157.

66. Herchenhorn D, Dias FL, Ferreira CG, Araújo CM, Lima RA, Small IA, Kligerman J. Impact of previous tracheotomy as a prognostic factor in patients with locally advanced squamous cell carcinoma of the larynx submitted to concomitant chemotherapy and radiation. ORL J OtorhinolaryngolRelatSpec. 2008;70(6):381-8.

67. Hashibe M1, Brennan P, Strange RC. Meta- and pooled analyses of GSTM1, GSTT1, GSTP1, and CYP1A1 genotypes and risk of head and neck cancer. Cancer Epidemiol Biomarkers Prev. 2003 Dec;12(12):1509-17.

68. Zhang Y, Chen W, Ji JF. GSTM1 null polymorphisms is associated with laryngeal cancer risk: a meta-analysis. Tumour Biol. 2014 Jul;35(7):6303-9

69. Brennan P, Lewis S, Hashibe M. Pooled analysis of alcohol dehydrogenase genotypes and head and neck cancer: a HuGE review. Am J Epidemiol. 2004 Jan 1;159(1):1-16.

70. Abbasi R, Ramroth H, Becher H. Laryngeal cancer risk associated with smoking and alcohol consumption is modified by genetic polymorphisms in ERCC5, ERCC6 and RAD23B but not by polymorphisms in five other nucleotide excision repair genes. Int J Cancer. 2009 Sep 15;125(6):1431-9.

71. Seijas-Tamayo R, del Barco-Morillo E, Fernández-Mateos J. Implicación de polimorfismos en genes reparadores del adn en la supervivencia libre de enfermedad (SLE) de pacientes diagnosticados de cáncer epidermoide de cabeza y cuello. O57, SEOM national congress 2015.

72. Rosen MP, Cheng X, Poh C. Use of allelic loss to predict malignant risk for low-grade oral epithelial dysplasia. Clin Cancer Res 2000; 6: 357.

73. Mao L, Lee JS, Fan YH. Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment. Nat Med 1996; 2: 682-5

74. Califano J, van der Riet P, Westra W. Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res. 1996 Jun 1;56(11):2488-92.

75. Brzoska PM, Levin NA, Fu KK. Frequent novel DNA copy number increase in squamous cell head and neck tumors. Cancer Res. 1995 Jul 15;55(14):3055-9.

76. Hermsen M, Guervós MA, Meijer G. New chromosomal regions with high-level amplifications in squamous cell carcinomas of the larynx and pharynx, identified by comparative genomic hybridization. J Pathol. 2001 Jun;194(2):177-82.

77. Callender T, el-Naggar AK, Lee MS. PRAD-1 (CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma. Cancer. 1994 Jul 1;74(1):152-8.

78. Ioachim E, Peschos D, Goussia A. Expression patterns of cyclins D1, E in laryngeal epithelial lesions: correlation with other cell cycle regulators (p53, pRb, Ki-67 and PCNA) and clinicopathological features. J Exp Clin Cancer Res. 2004 Jun;23(2):277-83.

79. Hibi K, Trink B, Patturajan M. AIS is an oncogene amplified in squamous cell carcinoma. Proc Natl Acad Sci U S A. 2000 May 9;97(10):5462-7.

80.Van Dyke DL, Worsham MJ, Benninger MS. Recurrent cytogenetic abnormalities in squamous cell carcinomas of the head and neck region. Genes Chromosomes Cancer. 1994 Mar;9(3):192-206.

81. Nawroz H1, van der Riet P, Hruban RH. Allelotype of head and neck squamous cell carcinoma. Cancer Res. 1994 Mar 1;54(5):1152-5.

82. Van der Riet P, Nawroz H, Hruban RH. Frequent loss of chromosome 9p21-22 early in head and neck cancer progression. Cancer Res. 1994 Mar 1;54(5):1156-8.

83. Kamb A, Gruis NA, Weaver-Feldhaus J. A cell cycle regulator potentially involved in genesis of many tumor types. Science. 1994 Apr 15;264(5157):436-40.

84. Okami K, Reed AL, Cairns P. Cyclin D1 amplification is independent of p16 inactivation in head and neck squamous cell carcinoma. Oncogene. 1999 Jun 10;18(23):3541-5.

85. Liggett WH Jr, Sewell DA, Rocco J, Ahrendt SA. p16 and p16 beta are potent growth suppressors of head and neck squamous carcinoma cells in vitro. Cancer Res. 1996 Sep 15;56(18):4119-23.

86. Yokoyama J, Shiga K, Sasano H. Abnormalities and the implication of retinoblastoma locus and its protein product in head and neck cancers. Anticancer Res. 1996 Mar-Apr;16(2):641-4.

87. Semczuk A, Marzec B, Roessner A. Loss of heterozygosity of the retinoblastoma gene is correlated with the altered pRb expression in human endometrial cancer. Virchows Arch. 2002 Dec;441(6):577-83. Epub 2002 Sep 25.

88. Xing EP, Yang GY, Wang LD. Loss of heterozygosity of the Rb gene correlates with pRb protein expression and associates with p53 alteration in human esophageal cancer. Clin Cancer Res. 1999 May;5(5):1231-40.

89. Rafferty M, Walker C, Husband D. Retinoblastoma gene abnormalities in early laryngeal cancer. Eur Arch Otorhinolaryngol. 2008 Jul;265 Suppl 1:S83-7.

90. Poeta ML, Manola J, Goldwasser M. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med. 2007 Dec 20;357(25):2552-61.

91. Bradford CR, Zhu S, Poore J. p53 mutation as a prognostic marker in advanced laryngeal carcinoma. Department of Veterans Affairs Laryngeal Cancer Cooperative Study Group. Arch Otolaryngol Head Neck Surg. 1997 Jun;123(6):605-9.

92. de Vicente C, Gutierrez LMJ, Zapatera AH. Prognostic significance of p53 expression in oral squamous cell carcinoma without neck noted metastases. Head Neck 2004; 26: 22.

93. Agrawal N, Frederick MJ, Pickering CR. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 333:1154–7.

94. Stransky N, Egloff AM, Tward AD. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011; 333:1157– 60.

95. Sun W, Gaykalova DA, Ochs MF. Activation of the NOTCH pathway in head and neck cancer. Cancer Res. 2014 Feb 15;74(4):1091-104

96. Krikelis D, Kotoula V, Bobos M. Protein and mRNA expression of notch pathway components in operable tumors of patients with laryngeal cancer. Anticancer Res. 2014 Nov;34(11):6495-503.

97. Velu TJ. Structure, function and transforming potential of the epidermal growth factor receptor. Mol Cell Endocrinol. 1990 May 7;70(3):205-16.

98. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res. 1993 Aug 1;53(15):3579-84.

99. Temam S, Kawaguchi H, El-Naggar AK. Epidermal growth factor receptor copy number alterations correlate with poor clinical outcome in patients with head and neck squamous cancer. J Clin Oncol. 2007;25(16):2164–2170

100. Dittmann K, Mayer C, Rodemann HP. Nuclear EGFR as novel therapeutic target: insights into nuclear translocation and function. Strahlenther Onkol. 2010;186(1):1–6

101. Maurizi M, Almadori G, Ferradina G. Prognostic significance of epidermal growth factor receptor in laryngeal squamous cell carcinoma. Br J Cancer 1996; 74: 1253-1257.

102. Nijkamp MM, Span PN, Terhaard CH. Epidermal growth factor receptor expression in laryngeal cancer predicts the effect of hypoxia modification as an additive to accelerated radiotherapy in a randomised controlled trial. Eur J Cancer. 2013 Oct;49(15):3202-9

103. Burtness B1, Goldwasser MA, Flood W; Eastern Cooperative Oncology Group. Phase III randomized trial of cisplatin plus placebo compared with cisplatin plus cetuximab in metastatic/recurrent head and neck cancer: an Eastern Cooperative Oncology Group study. J Clin Oncol. 2005 Dec 1;23(34):8646-54.

104. Sok JC, Coppelli FM, Thomas SM. Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. Clin Cancer Res. 2006 Sep 1;12(17):5064-73.

105. Bonner WM. gammaH2AX and cancer. Nature Reviews Cancer 8, 957-967 2008.

106. Guijarro MV, Leal JF, Fominaya J. MAP17 overexpression is a common characteristic of carcinomas. Carcinogenesis. 2007;28(8):1646-52.

107. Perez M, Praena-Fernandez JM, Felipe-Abrio B. MAP17 and SGLT1 protein expression levels as prognostic markers for cervical tumor patient survival. PLoS One. 2013; 8(2):e56169.

108. Guijarro MV, Vergel M, Marin JJ, Muñoz-Galván S, Ferrer I, Ramon y Cajal S, Roncador G, Blanco-Aparicio C, Carnero A. p38á limits the contribution of MAP17 to cancer progression in breast tumors. Oncogene. 2012; 31(41):4447-59.

109. Ivashkevich, A. et al. (2012) Use of the gamma-H2AX assay to monitor DNA damage and repair in translational cancer research. Cancer Lett, 327. 123-133.

110. Murray D, Rosenberg E. The importance of the ERCC1/ERCC4[XPF] complex for hypoxic-cell radioresistance does not appear to derive from its participation in the nucleotide excision repair pathway. Mutat Res 364: 217–226

111. Jun HJ, Ahn MJ, Kim HS. ERCC1 expression as a predictive marker of squamous cell carcinoma of the head and neck treated with cisplatin-based concurrent chemoradiation. British Journal of Cancer (2008) 99, 167–172

112. Lu B, Li J, Gao Q2. Laryngeal cancer risk and common single nucleotide polymorphisms in nucleotide excision repair pathway genes ERCC1, ERCC2, ERCC3, ERCC4, ERCC5 and XPA. Gene. 2014 May 25;542(1):64-8.

113. Santos CR, Rodríguez-Pinilla M, Vega FM. VRK1 signaling pathway in the context of the proliferation phenotype in head and neck squamous cell carcinoma. Mol Cancer Res 2006; 4: 177-185.

114. Thomas SJ, Snowden JA, Zeidler MP. The role of JAK/STAT signalling in the pathogenesis, prognosis and treatment of solid tumours. Br J Cancer. 2015; 113: 365-371.

115. O'Shea JJ, Schwartz DM, Villarino AV. The JAK-STAT pathway: impact on human disease and therapeutic intervention. Annu Rev Med. 2015; 66: 311-328.

116. Hosford SR, Miller TW. Clinical potential of novel therapeutic targets in breast cancer: CDK4/6, Src, JAK/STAT, PARP, HDAC, and PI3K/AKT/mTOR pathways. Pharmacogenomics and personalized medicine. 2014; 7: 203-215.

117. Furqan M, Akinleye A, Mukhi N. STAT inhibitors for cancer therapy. Journal of hematology & oncology. 2013; 6: 90.

118. Bournazou E, Bromberg J. Targeting the tumor microenvironment: JAK-STAT3 signaling. Jak-Stat. 2013; 2: e23828.

119. Turano C, Gaucci E, Grillo C. ERp57/GRP58: a protein with multiple functions. Cellular & molecular biology letters. 2011; 16: 539-563.

120. Choe MH, Min JW, Jeon HB. ERp57 modulates STAT3 activity in radioresistant laryngeal cancer cells and serves as a prognostic marker for laryngeal cancer. Oncotarget. 2015; 6: 2654-2666.

121. Gargalionis AN, Basdra EK. Insights in microRNAs biology. Curr Top Med Chem. 2013; 13: 1493-1502.

122. Weidhaas J. Using microRNAs to understand cancer biology. Lancet Oncol. 2010; 11: 106-107.

123. Yu X, Li Z. The role of microRNAs expression in laryngeal cancer. Oncotarget. 2015; 6: 23297-23305.

124. Olivieri F, Albertini MC, Orciani M. DNA damage response (DDR) and senescence: shuttled inflamma-miRNAs on the stage of inflamm-aging. Oncotarget. 2015; 6: 35509-35521.

125. Zhang SY, Lu ZM, Lin YF. miR-144-3p, a tumor suppressive microRNA targeting ETS-1 in laryngeal squamous cell carcinoma. Oncotarget. 2016.

126. Keck MK, Zuo Z, Khattri A, Stricker TP. Integrative analysis of head and neck cancer identifies two biologically distinct HPV and three non-HPV subtypes. Clin Cancer Res. 2015 Feb 15;21(4):870-81.

127. Chevalier D, Laccourreye O, Brasnu D, et al. Crycohyoidoepiglottopexy for glottic carcinoma with fixation o impaired motion of the true vocal cord: 5-year oncology results with 112 patients. Ann OtolRhinolLaryngol 1997; 106(5): 364-69.

128. Silver CE, Beitler JJ, Shaha AR, et al. Current trends in initial management of laryngeal cancer: the declining use of open surgery. Eur Arch Otorhinolaryngol. 2009; 266(9):1333-52.

129. Jørgensen K, Godballe C, Hansen O, et al. Cancer of the larynx: treatment results after primary radiotherapy with salvage surgery in a series of 1005 patients. ActaOncol. 2002; 41:69-76.

130. Fu K, Pajak T, Trotti A, et al. A Radiation Therapy Oncology Group (RTOG) phase III randomized study to compare hyperfractionation and two variants of accelerated fractionation to standard fractionation radiotherapy for head and neck squamous cell carcinomas: First report of RTOG 90-03. Int J Radiat Oncol Biol Phys. 2000;48:7–16.

131. P ster DG, Laurie SA, Weinstein GS, et al. American Society of Clinical Oncology clinical practice guideline for the use of larynx-preservation strategies in the treatment of laryngeal cancer. J Clin Oncol 2006; 24:3693.

132. Forastiere AA, Goepfert H, Maor M, et al. Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. N Engl J Med 2003; 349(22):2091-8.

133. Forastiere AA, Zhang Q, Weber RS, et al. Long-term results of RTOG 91-11: a comparison of three nonsurgical treatment strategies to preserve the larynx in patients with locally advanced larynx cancer. J ClinOncol. 2013 Mar 1;31(7):845-52.

134. Pignon JP, le Maître A, Maillard E, Bourhis J for the MACH-NC Collaborative Group. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomized trials and 17,346 patients. Radiotherapy and Oncology 2009; 92(1): 4-14.

135. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med. 2006 Feb 9;354(6):567-78.

136. Bonner JA, Harari PM, Giralt J, et al. Improved preservation of larynx with the addition of cetuximab to radiation for cancers of the larynx and hypopharynx. J Clin Oncol 2005 ASCO Annual Meeting Proceedings 2005; 23:5533.

137. Ang K, Zhang Q, Wheeler RH, et al: A phase III trial (RTOG 0129) of two radiation-cisplatin regimens for head and neck carcinomas (HNC): Impact of radiation and cisplatin intensity on outcome. J Clin Oncol 28:422s, 2010 (suppl; abstr 5507).

138. Ghi MG, Paccagnella A, Floriani I, Garavaglia D. Concomitant chemoradiation in locally advanced head and neck squamous cell carcinoma: A literature-based meta-analysis on the platinum concomitant chemotherapy. J Clin Oncol 29: 2011 (suppl; abstr 5534).

139. Strojan P, Vermorken JB, Beitler JJ, Saba NF, et al. Cumulative cisplatin dose in concurrent chemoradiotherapy for head and neck cancer: A systematic review. Head Neck. 2015 Mar 3.

140. Pointreau Y, Garaud P, Chapet S, et al. Randomized trial of induction chemotherapy with cisplatin and 5- fluorouracil with or without docetaxel for larynx preservation. J Natl Cancer Inst 2009; 101(7):498-506.

141. Lefebvre JL, Pointreau Y, Rolland F, et al. Induction chemotherapy followed by either chemoradiotherapy or bioradiotherapy for larynx preservation: the TREMPLIN randomized phase II study. J ClinOncol. 2013; 31(7):853-9.

142. Vermorken JB, Specenier P. Optimal treatment for recurrent/metastatic head and neck cancer. Ann Oncol. 2010 Oct;21 Suppl 7:vii252-61

143. Argiris A, Li Y, Forastiere A. Prognostic factors and long-term survivorship in patients with recurrent or metastatic carcinoma of the head and neck. Cancer. 2004 Nov 15;101(10):2222- 9.

144. Vermorken JB, Mesia R, Rivera F et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med 2008, 359:1116–1127

145. Gibson MK, Li Y, Murphy B, et al. Randomized phase III evaluation of cisplatin plus fluorouracil versus cisplatin plus paclitaxel in advanced head and neck cancer (E1395): an

intergroup trial of the Eastern Cooperative Oncology Group. J Clin Oncol. 2005 May 20;23(15):3562-7.

156. Burtness B, Goldwasser MA, Flood W, et al. Phase III randomized trial of cisplatin plus placebo compared with cisplatin plus cetuximab in metastatic/recurrent head and neck cancer: an Eastern Cooperative Oncology Group study. J Clin Oncol. 2005 Dec 1;23(34):8646-54.

147. Grau JJ, Caballero M, Verger E, et al. Weekly paclitaxel for platin-resistant stage IV head and neck cancer patients. Acta Otolaryngol. 2009 Nov;129(11):1294-9

148. Machiels JP, Haddad RI, Fayette J. Afatinib versus methotrexate as second-line treatment in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck progressing on or after platinum-based therapy (LUX-Head & Neck 1): an open-label, randomised phase 3 trial. Lancet Oncol. 2015 May;16(5):583-94.

149. Ferris RL, Blumenschein G Jr, Fayette J. Nivolumab for Recurrent Squamous-Cell Carcinoma of the Head and Neck. N Engl J Med. 2016 Nov 10;375(19):1856-1867.

150. Lefebvre JL, Ang KK, Larynx Preservation Consensus Panel. Larynx preservation clinical trial design: key issues and recommendations –-a consensus panel summary. Int J Radiat Oncol Biol Phys. 2009; 73(5):1293-303.

151. Ashraf MJ, Maghbul M, Azarpira N. Expression of Ki67 and P53 in primary squamous cell carcinoma of the larynx. Indian journal of pathology & microbiology. 2010; 53: 661-665.

152. Guijarro MV, Leal JF, Blanco-Aparicio C, Alonso S, Fominaya J, Lleonart M, Castellvi J, Ramon y Cajal S, Carnero A. MAP17 enhances the malignant behavior of tumor cells through ROS increase. Carcinogenesis. 2007; 28: 2096-2104.

153. Carnero A. MAP17 and the double-edged sword of ROS. Biochim Biophys Acta. 2012; 1826: 44-52.

154. Perez M, Peinado-Serrano J, Garcia-Heredia JM. Efficacy of bortezomib in sarcomas with high levels of MAP17 (PDZK1IP1). Oncotarget. 2016 Oct 11;7(41):67033-67046.

155. Matthaios D, Foukas PG, Kefala M. Gamma-H2AX expression detected by immunohistochemistry correlates with prognosis in early operable non-small cell lung cancer. OncoTargets and therapy. 2012; 5: 309-314.

156. Brunner AH, Hinterholzer S, Riss P. Expression of gamma-H2AX in endometrial carcinomas: an immunohistochemical study with p53. Gynecol Oncol. 2011; 121: 206-211.

157. Bartkova J, Horejsi Z, Koed K. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature. 2005; 434: 864-870.

158. Gorgoulis VG, Vassiliou LV, Karakaidos P. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature. 2005; 434: 907-913.

159. Bartek J, Bartkova J, Lukas J. DNA damage signalling guards against activated oncogenes and tumour progression. Oncogene. 2007; 26: 7773-7779.

160. Blagosklonny MV. Cell cycle arrest is not yet senescence, which is not just cell cycle arrest: terminology for TOR- driven aging. Aging (Albany NY). 2012; 4: 159-165.

161. Blagosklonny MV. Hypoxia, MTOR and autophagy: converging on senescence or quiescence. Autophagy. 2013; 9: 260-262.

162.Carnero A. Markers of cellular senescence. Methods Mol Biol. 2013; 965: 63-81.

163. Ruiz L, Traskine M, Ferrer I. Characterization of the p53 response to oncogene-induced senescence. PLoS ONE. 2008; 3: e3230.

164. d'Adda di Fagagna F. Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer. 2008; 8: 512- 522.

165. Pospelova TV, Demidenko ZN. Pseudo-DNA damage response in senescent cells. Cell Cycle. 2009; 8: 4112-4118.

166. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L. A DNA damage checkpoint response in telomere- initiated senescence. Nature. 2003; 426: 194-198.

167. Blasco MA. Telomere length, stem cells and aging. Nat Chem Biol. 2007; 3: 640-649.

168. Blagosklonny MV. Aging: ROS or TOR. Cell Cycle. 2008; 7: 3344-3354.

169. Fumagalli M, Rossiello F, Mondello C. Stable cellular senescence is associated with persistent DDR activation. PLoS One. 2014; 9: e110969.

170. Leontieva OV, Lenzo F, Demidenko ZN. Hyper-mitogenic drive coexists with mitotic incompetence in senescent cells. Cell Cycle. 2012; 11: 4642-4649.

171. Darzynkiewicz Z. When senescence masquerades as DNA damage: is DNA replication stress the culprit? Cell Cycle. 2009; 8: 3810-3811.

172. Nuciforo PG, Luise C, Capra M. Complex engagement of DNA damage response pathways in human cancer and in lung tumor progression. Carcinogenesis. 2007; 28: 2082-2088.

173. Bartkova J, Hamerlik P, Stockhausen MT. Replication stress and oxidative damage contribute to aberrant constitutive activation of DNA damage signalling in human gliomas. Oncogene. 2010; 29: 5095-5102.

174. Halicka HD, Zhao H, Li J. Potential anti-aging agents suppress the level of constitutive mTOR- and DNA damage- signaling. Aging (Albany NY). 2012; 4: 952-965.

175. Darzynkiewicz Z, Zhao H, Halicka HD. In search of antiaging modalities: evaluation of mTOR- and ROS/DNA damage-signaling by cytometry. Cytometry A. 2014; 85: 386-399.

176. Pankotai T, Hoffbeck AS, Boumendil C. DNA damage response in the absence of DNA lesions continued. Cell Cycle. 2009; 8: 4025-4026.

177. Leontieva OV, Blagosklonny MV. DNA damaging agents and p53 do not cause senescence in quiescent cells, while consecutive re-activation of mTOR is associated with conversion to senescence. Aging (Albany NY). 2010; 2: 924-935. doi: 10.18632/aging.100265.

178. Demidenko ZN, Blagosklonny MV. Growth stimulation leads to cellular senescence when the cell cycle is blocked. Cell Cycle. 2008; 7: 3355-3361.

179. Blagosklonny MV. Calorie restriction: decelerating mTOR- driven aging from cells to organisms (including humans). Cell Cycle. 2010; 9: 683-688.

180. Ferbeyre G, de Stanchina E, Lin AW. Oncogenic ras and p53 cooperate to induce cellular senescence. Mol Cell Biol. 2002; 22: 3497-3508.

181. Blagosklonny MV. Molecular damage in cancer: an argument for mTOR-driven aging. Aging (Albany NY). 2011; 3: 1130-1141.

182. Demidenko ZN, Zubova SG, Bukreeva EI. Rapamycin decelerates cellular senescence. Cell Cycle. 2009; 8: 1888-1895.

183. Anisimov VN, Zabezhinski MA, Popovich IG. Rapamycin extends maximal lifespan in cancer-prone mice. Am J Pathol. 2010; 176: 2092-2097.

184. Korotchkina LG, Leontieva OV, Bukreeva EI. The choice between p53-induced senescence and quiescence is determined in part by the mTOR pathway. Aging (Albany NY). 2010; 2: 344-352.

185. Pospelova TV, Leontieva OV, Bykova TV. Suppression of replicative senescence by rapamycin in rodent embryonic cells. Cell Cycle. 2012; 11: 2402-2407.

186. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006; 124:471-484. Sengupta S, Peterson TR, Sabatini DM. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Mol Cell. 2010; 40: 310-322.

187. Young AR, Narita M. Cell senescence as both a dynamic and a static phenotype. Methods Mol Biol. 2013; 965: 1-13.

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REVIEW

A genetic view of laryngeal cancer heterogeneity

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ABSTRACT

During the recent decades significant improvements in the understanding of laryngeal molecular biology allowed a better characterization of the tumor. However, despite increased molecular knowledge and clinical efforts, survival of patients with laryngeal cancer remains the same as 30 years ago. Although this result may not make major conclusions as preservation approaches were not broadly used until the time of database collection, it seems to be clear that there is still window for improvement. Although the cornerstone for laryngeal cancer eradication is to implement smoking cessation programs, survival progresses will be hopefully seen in the future. Introducing molecular biomarkers as predictive factors to determine which patients will benefit of preservation treatments may become one of the next steps to improve survival. Furthermore, the development of new therapeutic modalities joint to biomarkers to selectively apply such new therapy in these patients may help to define new modalities with improved survival. New inhibitors against Notch pathway, EGFR, VRK1 or DNA damage repair may become gold standard if we are able to identify patients that may benefit from them, either on survival or functional larynx preservation. It is the moment for an inflexion point on the way laryngeal cancer is clinically managed.

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Introduction

Squamous cell carcinoma of the head and neck (SCCHN) represents 4% of all cancers diagnosed worldwide, with >500.000 new cases recorded in 2008. Of them, 151.000 cases (130.000 men and 21.000 women) were laryngeal cancer. The estimated age-standardized world mortality rate is 2.3/100.000 habitants and 82.000 deaths were estimated in 2008 due to this cause.[1]

Cigarette smoking and alcohol consumption have been established as the major etiologic factors for laryngeal cancer. The risk may differ by tumor subsite in the larynx, with alcohol and tobacco abuse exerting a stronger effect on the supraglotic region.[2,3] Other occupational exposure risk factors as coal dust, hard-alloys dust and chlorinated solvents have also been implicated for laryngeal cancer.[4] The role of Human Papilomavirus (HPV) is well established for squamous cell carcinoma of the oropharynx but it remains unclear for laryngeal cancer.[5] In a systematic review that included more than 5 thousand SCCHNC HPV prevalence was 24%, lower than oropharyngeal cancer (35.6%). Of them, HPV16 was the most common type detecter in 16.6% of the laryngeal tumors.[6] However, the risk of developing laryngeal cancer in heavy smokers (>10 pack-year) seems to be independent to the HPV status.[7]

Furthermore, patients with laryngeal cancer are at risk of having second primary tumors due to chronic aerodigestive tract carcinogen exposure: 14% in 5 years, 26% in 10 years and 37% in 15 years.[8] On the other hand, there is evidence that suggest that consumption of fruits, vegetables, and carotene-containing foods may reduce the risk of laryngeal cancer.[9,10]

The main prognostic factor for overall survival (OS) is tumor staging, where node invasion is more relevant than tumor extension.[11] Other OS prognostic factors are patient's comorbidity, performance status-ECOG (PS),[12] persistent toxic consumption habits,[13] second primary tumor appearance[14] and primary tumor localization. In general, the supra and subglottic cancers have worse prognosis than glottic cancers, which 5-year survival is 82% for early stages possibly related to early detection.[15]

Moreover, PS,[12,16] node invasion[16,17] and localization[12] are prognostic factors for disease-free survival in conjunction with pathologic stage (pT),[17] surgical resection margins[16] and pre-treatment tracheotomy.[18] Moreover, T4 primary extension and more than 2 cm tumoral invasion of the base of the tongue were shown to be associated with increased salvage laryngectomy in the Veterans study.[19]

Molecular biology

Genetic susceptibility

Emerging phenotypic and genotypic data support the idea of genetic susceptibility for SCCHN. In an historical cohort study the relative risk (RR) for SSCHN was 7.89 (95% confidence interval -CI-1.50 to 41.6) in first degree relatives of patients with multiple primary head and neck cancer.[19] In a more recent

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pooled analysis with 8.967 SCCHN cases and 13.627 controls, having a family history of SCCHN in first-degree relative increased the risk of SCCHN 1.7, which was higher when the affected relative was a sibling (OR= 2.2, 95% CI 1.6–3.1) rather than a parent (OR = 1.5, 95% CI 1.1–1.8) and for more distal SCCHN anatomic sites (hypopharynx and larynx).[20] Genetic polymorphisms variants in tobacco carcinogen and alcohol metabolism genes may increase SCCHN risk. GSTM1 null genotype appears to confer increased SCCHN and particularly laryngeal increased risk (OR = 1.22, 95% CI 1.1–1.36).[21,22] The variant Val allele of the CYP1A1 Ile462Val polymorphism is another consistent susceptibility maker for SCCHN, with a 35% increased risk in a meta-analysis of 12 studies.[22] Presenting fast metabolizing alleles for alcohol dehydrogenase (ADH), ADH1B and ALDH2 genes, resulted in increased acetyladehyde levels and associated with HNSCC significantly interacting with alcohol consumption.[23] In a case-control study single nucleotide polymorphisms (SNPs) in nucleotide excision repair (NER) genes such as ERCC5, ERCC6 and RAD23B could modify laryngeal cancer risk. In particular, ERCC6 showed a decreased risk, while ERCC5 and RAD23B increased it.[24] Furthermore, XPD and ERCC1 may be associated to poor disease free survival (DFS) in SCCHN, suggesting a significant role of NER in these tumors.[25]

Cytogenetic alterations

Malignant progression may be associated to particular chromosomal alterations. For instance, early changes at 3p, 4q, 8p, 9p, 11q, 13q and 17p have been observed in leukoplakias while loss of heterozygosity (LOH) of 9p21 is a common genetic event in oral premalignancies.[26,27] Califano et at described a model of carcinogenesis in 1996, but it seems to be more the accumulation rather than the order of genetic events what determines tumoral progression (Fig. 1).[28]

Amplifications/loss of heterozygosity

Amplification in a variety of key genes have been described in SCCHN, such as of 3q, 3q24-qter, 5p, 8q23–24, 11q13, 11q14–22, 18p, 18q11.2, and 19q among others.[29,30] The critical proto-oncogene Cyclin D1 is amplificated within the 11q13 region and may be a marker of progression in primary SCCHN.[31] Cyclin D1 overexpression has been reported in laryngeal carcinoma and might be implicated in regulating cell proliferation by the critical G1/S checkpoint.[32] P63 is a p53 homolog and a potential oncogene in squamous cell cancer that has been found in the distal arm of 3q.[33]

Loss of chromosomes 3p, 5q, 8p, 9p, 18q and 21q are commonly identified as well, where loss of 18q could indicate poor prognosis tumors.[34] The most commonly deleted region in SCCHN is located at chromosome 9p21–22.[35] It occurs in the majority of invasive tumors and is present at a high frequency in early premalignant lesions, including dysplasia and carcinoma in situ.[36] P16 or cyclin-dependent kinase inhibitor 2 (CDKN2-) is the tumor suppressor gene contained within this critically deleted region and is a potent inhibitor of cyclin D1/CDK4.[37] However, p16 amplification is independent of cyclin D1 inactivation in SCCHN.[38] It is possible that a second tumor suppressor gene resides at 9p21. An alternative rating frame (ARF) RNA transcript for p16 codes for a protein through an alternate reading frame. Introduction of p16 or p16ARF into head and neck cancer cell lines results in potent growth suppression.[39] Loss of heterozygosity (LOH) at the Retinoblastoma (Rb) locus in 13q14 has been found in around 14% of SCCHN, leading to Rb protein inactivation and tumor progression.[40] Rb LOH is correlated with altered pRB expression in endometrial and esophageal cancer[41,42] but same results could not be found for laryngeal cancer.[43]

TP53

Mutations in p53 are one of the most frequent abnormalities in SCCHN and can be observed in severe dysplasia. TP53 mutations are found in 39–53% of SCCHN tumors and in 56.7% of laryngeal carcinomas.[44,45] P53 mutations have been demonstrated to be related to poor survival in different publications by using microarray technology,[44] immunohistochemistry (IHC)[46] or single-strand conformational polymorphism (SSCP) analysis followed by DNA sequencing. However, there is not necessarily correlation

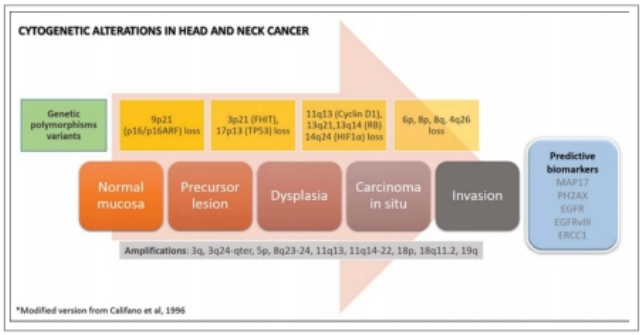

Figure 1. Frequent cytogenetic alterations in head and neck cancer.

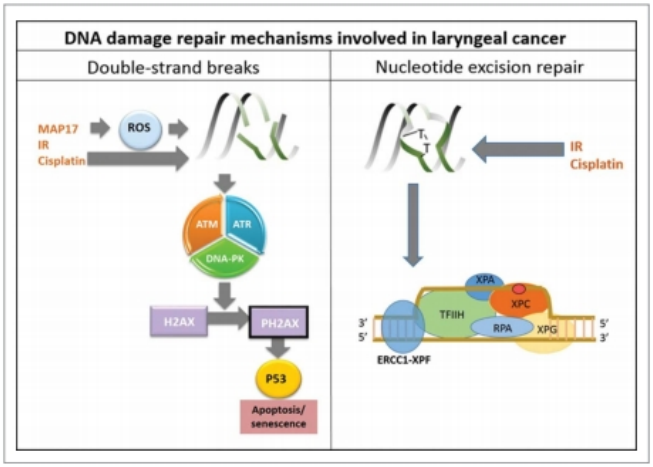

Figure 2. Mechanism of response to DNA damage in laryngeal cancer.

between IHC and SSCP results[45]; moreover, decrease in survival can vary if the mutation is disruptive or nondisruptive.[44] These controversies may explain differences as a prognostic factor found so far.

NOTCH

Inactivating mutations of NOTCH1 have been found in 10–15% of SCCHN, being the second most frequently mutated gene after TP53.[47,48] NOTCH signaling pathway has been linked to multiple biological functions, such as regulation of self-renewal capacity, cell cycle exit, and survival. In SCCHN, several of the NOTCH family mutations encode inactivating mutations, suggesting a tumor suppressor function.[49] In a study that included 289 patients with laryngeal carcinoma Notch3 was associated with unfavorable disease-free survival and overall survival.[50]

EGFR/EGFRvIII

The EGFR pathway is involved in cell transformation through autocrine overproduction of epidermal growth factor/transforming growth factor α (EGF/TGFα) and overexpression of EGFR by gene amplification or altered transcriptional mechanisms.[51] Production of TGFα and EGFR mRNA has been found in normal mucosa of patients at risk for a primary or secondary SCCHN suggesting these changes to be early markers of carcinogenesis.[52]

In up to 90% of SCCHN, increased expression of EGFR is observed which is associated with advanced stage, poor survival and resistance to treatments.[53,54] Similarly, EGFR overexpression in laryngeal carcinoma has also been linked to poor survival[55] and also as a predictive biomarker for radiotherapy treatment.[56] In a phase III trial comparing cisplatin with or without the monoclonal antibody cetuximab for metastatic/recurrent SCCHN patients, it was seen that doses of cetuximab may need to be adjusted for patients with very high levels of EGFR to achieve better response.[57] The EGFRvIII mutant variant has been found in up to 40% of SCCHN and it seems to contribute to cancer growth and resistance to EGFR targeting. EGFRvIII is seen in cells that overexpress wild-type EGFR suggesting that mutations are a later event caused by rapid proliferation induced by EGFR overexpression.[58]

DNA damage repair biomarkers

As laryngeal cancer respond to treatments based on platinum and radiotherapy that result in DNA damage, biomarkers implicated in the nucleotide excision repair (NER) and the double strand breaks repair might have a significant role. DNA Double break streams (DBS) can be originated by drugs and ionizing radiation but also by increasing the levels of reactive oxygen species (ROS)[59] through MAP17 activation. MAP17 is a small non-glycosylated membrane protein overexpressed in carcinomas. MAP17 expression increases the levels of reactive oxygen species (ROS) in cells which may account for some of the increased tumoral properties. In turn, further increase of ROS might switch the balance toward apoptosis.[60,61] Patients with laryngeal cancer and high levels of MAP17 have been found correlation to have improved overall survival and laryngoesophageal dysfunction-free survival rates.[62] DNA DBS lead to activation of 3 kinases, ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR) and DNA-PK which phosphorylate γH2AX, a component of the histone octamer in nucleosomes (Figure 2). PH2AX is involved in recruiting DNA repair proteins in response to DSB.[59] High pH2AX expression was associated with prolonged LDS in patients with laryngeal cancer.[63]

The NER pathway guard the integrity of the genome by recognizing and removing DNA cross-links caused by cisplatin or radiation mainly driven by the excision repair cross-complementation group 1 (ERCC1).[64] In locally advanced SCCHN low expression of ERCC1 was an independent predictor factor for prolonged in patients treated with cisplatin-based CCRT;

however just 18% of patients had laryngeal carcinoma.[65] Furthermore, in a case-control study focused on laryngeal cancer, ERCC1 rs11615 and ERCC5 rs17655 polymorphisms were associated with increased risk of developing laryngeal cancer.[66]

Vaccinia-related kinase-1 (VRK1) protein

VRK1 protein belongs to a family of 3 protein kinases implicated in regulation of cell proliferation by phosphorylation of p53 and cooperation with c-Jun and ATF2. VRK1 expression is activated by E2F and inhibited by p16 and Rb. In SCCHN, VRK1 could be a significant control mechanism of the cell cycle, particularly in G1-S phase.[67]

Stat3 pathway

Stat3 (signal transducer and activator of transcription 3) is a transcription factor that responds to cytokines and growth factor receptor activation.[68] Constitutive activation of the pathway in response to deregulate upstream signals is commonly observed in diverse cancers including dead and neck and laryngeal tumors.[69-71] This constitutive activation of the pathway has been involved in proliferation and survival of tumors as well as resistance to chemo and radiotherapy.[70,71] Recent works indicate that STAT signaling also contributes to therapy resistance by modulating also the microenvironment.[72] ERp57 (GRP58) is a chaperone that regulated proper folding of glycoproteins.[73] ERp57 is associated with tumor progression ans has been described to modulate STAT 3 activity, thus regulating radioresistance in laryngeal cancer.[74] Accordingly, ERp57 has been described as a poor prognosis factor.

Micro RNAs

MicroRNAs (miRs) play important roles in many pathological alterations regulating important cellular and physiological processes such as cell proliferation, differentiation, metabolism, apoptosis, autophagy and intercellular communications.[75,76] It has been considered that miRs regulate around 60% of genes in the numan genome. The analysis of miR expression variation in Laryngeal cancer by a variety of techniques including broadly used miR microarrays and massive sequencing have shown a large variety of miRs deregulated in laryngeal cancer, many of them with diagnosis or prognosis value (reviewed in[77])[78,79] (Table 1). These miRs behave as oncogenes (oncomirs) or tumor suppressorS in laryngeal cancer according the effect of the target. However this simplistic analysis is commonly more complicated since one miR can target several genes and several miRs can target the same gene thus providing some synergistic effects. Interestingly, several miRs have been associated with therapeutic resistance in laryngeal cancer, thus providing worse prognosis (Table 1).

Treatment

Early stage disease (Stages I and II)

Total laryngectomy was the gold standard treatment by the 1980s with the subsequent loss of speech and airway patency.[80] Consequently, treatment aims changed in order to improve patient's quality of life through larynx sparing approaches. Currently, early stages are treated with either surgery or radiotherapy (RT) as they have been accepted to have similar effectiveness. However, both treatments have not been compared in a randomized trial so far. Reported 5-year OS is typically reported as 70 to 90%.[81,82]

Locally advanced (Stages III and IVA/B)

Advanced disease requires multimodal approach, usually a combination of chemotherapy (CT) or biotherapy (B) plus RT. Although functional organ sparing approaches permit larynx preservation, they do not provide a survival advantage over total laryngectomy.[83] Three sparing approaches are accepted: RT, bio or chemotherapy with concomitant radiotherapy (B/CT RT) and induction CT (ICT) followed by RT with or without B/CT.

CTRT with concurrent cisplatin showed higher preservation rates compared to other 2 arms with RT alone or induction cisplatin plus fluorouracil followed by RT (88% versus –vs- 70% and 75%, respectively) with similar 2 and 5 year survival.[84] Later, a 10-year follow-up publication confirmed that the arms that included ICT improved laryngectomy-free survival (LFS). Contrary to preservation rates, LFS includes not just the need of salvage laryngectomy but also speech and swallowing quality. It is, therefore, more similar to what we currently understand as larynx preservation.[85] A subsequent meta-analysis for locally advanced larynx cancer found that adding CT concomitant

Table 1. Summary of miRNAs relevant in laryngeal cancer. Adapted from Yu and Li, 2015.[10] ND: not determined.

miRNAs	Target	Prognosis
Upregulated		
miR-16	Zyxin	ND
miR-19a	TIMP2	Poor survival, Lymph node metastasis
miR-21	BTG2	Poor survival, poor differentiation and Lymph node metastasis
miR-27a	PLK2	ND
miR-106b	RUNX3	Poor survival, poor differentiation and Lymph node metastasis
miR-129-5p	APC	ND
miR-155	SOCS1, STAT3	Poor differentiation and TNM stage
miR-1297	PTEN	ND
Downregulated		
miR-1	FN1	ND
miR-24	S100A8	ND
miR-144-3p	ETS-1	Poor prognosis
miR-34a	Survivin	Good prognosis
hsa-miR-34c	C-Met	ND
miR-126	Camsap1	ND
miR-139	CXCR4	ND
miR-203	ASAP1	Good survival, inverse to TNM and grade of differentiation.
miR-206	VEGF	Good survival, inverse to TNM and clinical stage.
miR-299-3p	hTERT	ND
miR-370	Fox-M1	ND
miR-519a	HuR, COX-2	ND
miR-874	HDAC1	ND

Table 2. Summary of the most significant clinical trials involving locally advanced laryngeal cancer treatment.

Trial	Phase, patients	Design	Inclusion criteria	Primary end point	Primary end point result	Secondary end points	Statistics	Others
Veterens1991	III, 332P	CFx3->RT (if response to CF) vs S->RT	III/IV glottic or supraglotic	Preservation rate	Preservation 64% vs 0%	31% CR, 54% PR. 68% 2-year OS in both arms	All randomized P included	More local recurrences with fewer distant mets in the CF arm.Latter analysis showed T4 to have better outcomes with surgery.*
Forastiere,RTOG91-112013	III, 518P	3 arms: CF->RT vs cis-RT vs RT	III/IV glottic or supraglottic**	2-y LFS	Cis-RT 88%:CF->RT 75, RT 70%	LRC cis-RT 78%, CF->RT 61%, RT 56%. Similar OS rates	Designed to test if cis-RT or RT resulted in higher rates of preservation than CF->RT. Detect 15% difference, type I errOR=0.05, type II=0.20.	
Forastiere, RTOG91-112013	Previous trial 10-y update	3 arms: CF->RT vs cis-RT vs RT	III/IV glottic or supraglottic**	10-y LFS	Cis-RT 23.5%, CF->RT 28.9%, RT 17.2% (cis-RT vs CF->RT HR=1.05, p.68)	Laryngeal preservation: cis-RT vs CF->RT HR=0.58, p.005.LRC: cis-RT vs CF->RT HR=0.66, p.006		The benefit of cis-RT for LFS is not maintained, but secondary end-points are. No collected information about gastrostomy tube need.
Bonner 2006	III, 424P	RT with or without cetuxi	III/IV oropharynx, hypopharynx, and larynx	LRC	Cetuxi-RT vs RT HR=0.68, p.005, (2y= 50 vs 41 %, p.005)	cetuxi-RT OS HR=0.74, p.03	Planned 1y-LRC RT 44%, cetuxi-RT 57%. 90% power, 5% 2 sided LRC	Possible major benefit for patients with oropharynx cancer
Bonner 2010	Previous trial 5-y update	RT with or without cetuxi	III/IV oropharynx, hypopharynx, and larynx	LRC/OS	5-year LRC and DFS were not reported.OS in the cetuxi-RT vs. RT HR 0.73, p=0.018			P with G2-4 cetuxi-induced acneiform rash had better OS that patients with mild rash.
Vermorken, 2007	III, 358P	DCF vs CFx4 cycles-> RT if no PD	III/IV SCCHN (with no M1)	PFS	DCF group HR 0.72, p=0.007	Reduction in the risk of death 27%	90% power to detect 15% differences in the 1-y survival rate	Ciprofloxacin as primary prophylaxis. G-CSF as secondary.
Pignon, 2009	Meta-Analysis, 16485P	Locoregional treatment +/− CT.	Non-metastatic HCN	OS	DFS,LRC, and distant failure	Absolute benefit for CT 4.5% at 5 years.For concomitant trials, HR 0.81 p.0.0 and absolute benefit 6.5% at 5 years.Concurrent CDDP was identified as the most effective agent.		
Pointreau,GORTEC 2000-01 2009	III, 213P	CF vs DCFx3-> CTRT***	III/IV larynx/ hypopharynx	Preservation rate	DCF 70.3% vs PF 57.5%, p=0.03	ORR DCF 80.0% vs PF 59.2% p=0.002	70% power to detect 15% improvement preservation. Type I error was 0.05*	Ciprofloxacin as primary prophylaxis. G-CSF as secondary.

Table 2. (*Continued*)

Trial	Phase, patients	Design	Inclusion criteria	Primary end point	Primary end point result	Secondary end points	Statistics	Others
Koutcher, 2011	Retrospective, 174P	Cis-RT vs cetuxi-RT	III/IV SCCHN	Cis-RT superior for 2-y locoregional failure (5.7% *v* 39.9%; *p*.001), failure-free survival (87.4% *v* 44.5%; *p*.001), and OS (92.8% *v* 66.6%; *p*.001).				Lack of HPV data. Possibly biased as P in cetuxi-RT were older and with renal impairment.
Lefebvre, Tremplin2013	II, 153P	DCFx3-> cetuxi-RT or cis-RT	Locally advanced larynx/ hypopharynx	3-m preservation	No differences:Cis-RT 95% vs cetuxi-RT 93%.	Preservation 87% cis-RT vs 82% cetuxi-RT; 18-m OS: 92% vs 89%.	Studied on randomly assigned population (76%) so inflated. 90% power; 5% type I error. No stratification.	Local recurrence: Cetuxi 21% vsCis 8% (p=0.08). None of the arms could show any substantial benefit compared with the GORTEC 2000–01 trial.

*Wolf GT. Reexamining the treatment of advanced laryngeal cancer: the VA laryngeal cancer study revisited. Head Neck 2010;32:7–14.
**T1 and high-volume T4 were excluded: invasion >1 cm into de base of tongue or penetration through caRTilage.
***Chemotherapy (cisplatin, carboplatin, and 5-fluorouracil or a combination of 2 drugs) during radiotherapy was allowed for all patients who were treated at the same institute, according to its practice.
P= patients. SCCHN: squamous cell carcinoma of the head and neck. CF: cisplatin, 5-Fluorouracil. RT: radiotherapy. S: surgery. CR: complete response. PR: partial response. PD: progression disease. OS: overall survival. Mets: metastases. Cis: cisplatin. Y: years. M: months. DFS: disease-free survival. LFS: laryngectomy-free survival. LCR: locoregional control. Cetuxi: cetuximab. PFS: progression-free survival. DCF: docetaxel, cisplatin, 5-Fluorouracil. ORR: overall response rate

with RT led to a benefit of 6.5% absolute improvement in 5-year OS.[86]

In a phase III trial, patients with advanced head and neck cancer who received combination treatment with RT and cetuximab, demonstrated a statistically significant advantage with respect to locoregional control (LRC) and survival compared to patients treated with radiation alone.[87] A subset analysis of the patients with hypopharyngeal and laryngeal tumors showed a preservation hazard ratio (HR) of 0.62 in the cetuximab arm but this was not statistically significant.[88]

Regarding ICT, the Veterans study demonstrated 64% preservation larynx rate without worsening survival in the ICT-RT arm compared with surgery-RT.[89] More efficacious induction regimens were further developed and the docetaxel, cisplatin, and 5-Fluorouracil (DCF) schedule became the standard treatment, preserving the larynx 15% more than cisplatin and 5-Fluorouracil.[89]

DCF induction followed by radical RT with concomitant cetuximab or cisplatin showed similar larynx preservation rates in both arms in a phase II randomized trial. Endpoints were evaluated on the randomly assigned population only, which represented 76% of the patients included in the trial so they were inflated. None of the arms could show any substantial benefit compared with the GORTEC 2000–01 trial, and therefore there is still not evidence enough for sequential therapy in this setting.[90]

The optimal dose of cisplatin during RT remains still unclear. This was studied as a subset analysis of the RTOG 0129 phase III trial, which compared accelerated concomitant boost vs standard RT fractionation. Receiving one cycle of 100 mg/m2 cisplatin was associated with worse OS, PFS and locoregional failure compared to 2 and 3 cycles. The third dose of cisplatin had no impact on OS or PFS compared with 2 cycles, but was associated with better LRC rate.[91] A meta-analysis presented in 2011 also suggested that there could be a dose/efficacy relation for concomitant cisplatin total dose. In this analysis, no advantage in OS was observed between CRT with cisplatin total dose <150mg/ m2 and RT alone. No difference in OS was observed between cisplatin high dose (300mg/m2) (HR 0.59, 95% CI 0.46–0.74) and cisplatin <300mg/m2 plus 5-fluorouracil (HR 0.59, 95% CI 0.45– 0.77) when compared to RT alone.[92] Finally, a systematic review showed that in 6 definitive radiotherapy phase III trials there was a statistically significant association between cumulative cisplatin dose, and overall survival benefit for higher doses.[93] In summary, 2 or 3 courses of 3-weekly cisplatin could be considered the optimal dose for concurrent CTRT and equivalent doses of carboplatin have also been accepted by expert panels.

Metastatic or recurrent disease (Stage IVC)

Despite treatment progress, locoregional recurrences and distant metastases occur in 20–30% of patients with SSCHN, and just few of them benefit from salvage surgery or re-irradiation. Treatment options for metastatic disease include supportive care, single agent or a chemotherapy combination with or without targeted agents.[94] Pronostic factors of long-term survivors in metastatic SCCHN patients treated with pratinum-based chemotherapy were identified in the E1395 and E1393 randomized trials of the Eastern Cooperative Oncology Group (ECOG) and include: tumor cell differentiation, ECOG performance status (PS), weight loss, location of the primary tumor and prior radiotherapy.[95]

The EXTREME trial showed that the combination of platinum-5Fluorouracil and cetuximab as first-line treatment in SSCHN improved OS, PFS and response rate with no decreased quality of life compared to the same schedule without the monoclonal antibody. Therefore, it has become the standard of treatment for patients with good PS.[96] Less intensive schedules include de combination of cisplatin-paclitaxel or cisplatin-5Fluorouracil, as they have been shown to achieve similar overall survival.[97] Other regimens as cisplatin plus cetuximab improved response rate in a phase III trial but with no benefit of PFS which was the primary end-point.[98] Single agent activity remains poor but it may be an alternative when other options are exhausted. Classical drugs as methotrexate, cisplatin, 5-fluorouracil (5-FU) and bleomyin have shown responses of short duration (3–5 months) and 15–30% tumor reduction.[94] Of the more recent agents, taxanes (docetaxel and paclitaxel) improved response rates up to 43% in platinum resistant patients.[99] Furthermore, Afatinib is an irreversible ERBB family blocker with significant results in the second-line setting. In a recent phase III trial it was compared to methotrexate in patients that had progressed to platinum-based regimens (including also cetuximab) and the primary end-point PFS was superior (2.6 m vs 1.7 m, $p = 0.03$). However, G3/4 toxicities were also higher in the afatinib arm.[100]

Conclusions

During the recent decades significant improvements in the understanding of laryngeal molecular biology allowed a better characterization of the tumor. SCCHN is considered now to be the final stage of a multi-step process in which LOH, amplifications, deletions, up and down-regulation oncogenes or tumor-suppressor genes take part. However, despite knowledge improvements and clinical efforts, survival of patients with laryngeal cancer remains unsatisfactory, and 5-year OS was reported to drop from 67.4% in 1985 to 61.9% in 2007.[101] Although this result may not make major conclusions as preservation approaches were not broadly used until the time of database collection, it seems to be clear that there is still window for improvement. Actually just the addition of cetuximab to the combination of platinum and 5-Fluorouracil has improved OS and changed clinical practice in recent times.[84] And new treatments are currently on study (Table 2), some of them with promising results as afatinib[100] or cabacitaxel.[102] Although the cornerstone for laryngeal cancer eradication is to implement smoking cessation programs, survival progresses will be hopefully be seen in the future. Introducing molecular biomarkers as predictive factors to determine which patients will benefit of preservation treatments may become one of the next steps to improve survival. Furthermore, the development of new therapeutic modalities joint to biomarkers to selectively apply such new therapy in these patients may help to define new modalities with improved survival. New inhibitors against Notch pathway, EGFR, VRK1 or DNA damage repair may become gold standard if we are able to identify patients that

may benefit from them, either on survival or functional larynx preservation. It is the moment that we perform an inflexion point on the way to clinically manage laryngeal cancer.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

[1] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; 127:2893-917; PMID:21351269; http://dx.doi.org/10.1002/ijc.25516.

[2] Guenel P, Chastang JF, Luce D, Leclerc A, Brugère J. A study of the interaction of alcohol drinking and tobacco smoking among French cases of laryngeal cancer. J Epidemiol Community Health 1988; 42:350-4; http://dx.doi.org/10.1136/jech.42.4.350.

[3] Falk RI, Pickle LW, Brown LM, Mason TJ, Buffler PA, Fraumeni JF Jr. Effects of smoking and alcohol consumption on laryngeal cancer risk in coastal Texas. Cancer Res 1989; 49:4024-9; PMID:2736543.

[4] Shangina O, Brennan P, Szeszenia-Dabrowska N, Mates D, Fabiánová E, Fletcher T, t'Mannetje A, Boffetta P, Zaridze D. Occupational exposure and laryngeal and hypopharyngeal cancer risk in central and eastern Europe. Am J Epidemiol 2006; 164(4):367-75; PMID:16801374; http://dx.doi.org/10.1093/aje/kwj208.

[5] Torrente MC, Rodrigo JP, Haigentz M Jr, Dikkers FG, Rinaldo A, Takes RP, Olofsson J, Ferlito A. Human papillomavirus infections in laryngeal cancer. Head Neck 2011; 33(4):581-6; PMID:20848441; http://dx.doi.org/10.1002/hed.21421.

[6] Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomarkers Prev 2005 Feb; 14(2):467-75; http://dx.doi.org/10.1158/1055-9965.EPI-04-0551.

[7] Ang K, Zhang Q, Wheeler RH, Rosenthal DI, Nguyen-Tan F, Kim H, Lu C, Axelrod RS, Silverman CI, Weber RS. A phase III trial (RTOG 0129) of two radiation-cisplatin regimens for head and neck carcinomas (SCCHN): Impact of radiation and cisplatin intensity on outcome. J Clin Oncol 2010; 28:15s (suppl; abstr 5507); http://dx.doi.org/10.1200/JCO.2010.28.3085.

[8] Gao X, Fisher SG, Mohideen N, Emami B. Second primary cancers in patients with laryngeal cancer: a population-based study. Int J Radiat Oncol Biol Phys 2003; 56:427-35.

[9] Mackerras D, Buffler PA, Randall DE, Nichaman MZ, Pickle LW, Mason TJ. Carotene intake and the risk of laryngeal cancer in coastal Texas. Am J Epidemiol 1988; 128:980-8; PMID:3189297.

[10] La Vecchia C, Negri E, D'Avanzo B, Franceschi S, Decarli A, Boyle P. Dietary indicators of laryngeal cancer risk. Cancer Res 1990; 50:4497-500; PMID:2369728.

[11] Sessions D. Surgical pathology of cancer of the larynx and hypopharynx. Laryngoscope 1976; 132:504-7.

[12] Singh B, Bhaya M, Stern J, Roland JT, Zimbler M, Rosenfeld RM, Har-El G, Lucente FE. Validation of the Charlson comorbidity index in patients with head and neck cancer: a multi-institutional study. Laryngoscope 1997; 107(9 11 Pt1):1469-75; PMID:9369392; http://dx.doi.org/10.1097/00005537-199711000-00009.

[13] Mayne ST, Cartmel B, Kirsh V, Goodwin WJ Jr. Alcohol and tobacco use prediagnosis and postdiagnosis, and survival in a cohort of patients with early stage cancers of the oral cavity, pharynx, and larynx. Cancer Epidemiol Biomarkers Prev 2009; 18 (12):3368-74; PMID:19959684; http://dx.doi.org/10.1158/1055-9965.EPI-09-0944.

[14] Di Martino E, Sellhaus B, Hausmann R, Minkenberg R, Lohmann M, Esthofen MW. Survival in second primary malignances of patients with head and neck cancer. J LaryngolOtol 2002; 116 (10):831-8.

[15] American Joint Committee on Cancer. Larynx. In: *AJCC Cancer Staging Manual*. 7th ed. New York, NY: Springer; 2010: 57-62.

[16] Zhang SY, Lu ZM, Luo XN, Chen LS, Ge PJ, Song XH, Chen SH, Wu YL. Retrospective analysis of prognostic factors in 205 patients with laryngeal squamous cell carcinoma who underwent surgical treatment. PLoS One 2013 Apr 4; 8(4):e60157; http://dx.doi.org/10.1371/journal.pone.0060157.

[17] Yilmaz T, Hoşal S, Ozyar E, Akyol F, Gürsel B. Post-operative radiotherapy in advanced laryngeal cancer: effect on local and regional recurrence, distant metastases and second primaries. J Laryngol Otol 2005 Oct; 119(10):784-90.

[18] Herchenhorn D, Dias FL, Ferreira CG, Araújo CM, Lima RA, Small IA, Kligerman J. Impact of previous tracheotomy as a prognostic factor in patients with locally advanced squamous cell carcinoma of the larynx submitted to concomitant chemotherapy and radiation. ORL J Otorhinolaryngol Relat Spec 2008; 70(6):381-8; http://dx.doi.org/10.1159/000163034.

[19] Foulkes WD, Brunet JS, Sieh W, Black MJ, Shenouda G, Narod SA. Familial risks of squamous cell carcinoma of the head and neck: retrospective case-control study. BMJ 1996 Sep 21; 313(7059):716-21; http://dx.doi.org/10.1136/bmj.313.7059.716.

[20] Negri E, Boffetta P, Berthiller J, Castellsague X, Curado MP, Dal Maso L, Daudt AW, Fabianova E, Fernandez L, Wünsch-Filho V, et al. Family history of cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. Int J Cancer 2009; 124(2):394-401; PMID:18814262; http://dx.doi.org/10.1002/ijc.23848.

[21] Hashibe M, Brennan P, Strange RC, Bhisey R, Cascorbi I, Lazarus P, Oude Ophuis MB, Benhamou S, Foulkes WD, Katoh T, et al. Meta- and pooled analyses of GSTM1, GSTT1, GSTP1, and CYP1A1 genotypes and risk of head and neck cancer. Cancer Epidemiol Biomarkers Prev 2003 Dec; 12(12):1509-17.

[22] Zhang Y, Chen W, Ji JF, Wang ZY, Wu MH, Zhang K, Wang QP. GSTM1 null polymorphisms is associated with laryngeal cancer risk: a meta-analysis. Tumour Biol 2014 Jul; 35(7):6303-9; http://dx.doi.org/10.1007/s13277-014-1828-x.

[23] Brennan P, Lewis S, Hashibe M, Bell DA, Boffetta P, Bouchardy C, Caporaso N, Chen C, Coutelle C, Diehl SR, et al. Pooled analysis of alcohol dehydrogenase genotypes and head and neck cancer: a HuGE review. Am J Epidemiol 2004 Jan 1; 159(1):1-16; http://dx.doi.org/10.1093/aje/kwh003.

[24] Abbasi R, Ramroth H, Becher H, Dietz A, Schmezer P, Popanda O. Laryngeal cancer risk associated with smoking and alcohol consumption is modified by genetic polymorphisms in ERCC5, ERCC6 and RAD23B but not by polymorphisms in five other nucleotide excision repair genes. Int J Cancer 2009 Sep 15; 125(6):1431-9; http://dx.doi.org/10.1002/ijc.24442.

[25] Seijas-Tamayo R, del Barco-Morillo E, Fernández-Mateos J, Cieza-Borrella C, Adansa Klain JC, Marcos Sánchez RA, Guillén Sacoto MC, González-Sarmiento R, Cruz-Hernández JJ. (2015, Oct). Implicación de polimorfismos en genes reparadores del adn en la supervivencia libre de enfermedad (SLE) de pacientes diagnosticados de cáncer epidermoide de cabeza y cuello. XV National Spanish Society of Medical Oncology Congress, O57. (Personal communication)

[26] Rosen MP, Cheng X, Poh C, Lam WL, Huang Y, Lovas J, Berean K, Epstein JB, Priddy R, Le ND, et al. Use of allelic loss to predict malignant risk for low-grade oral epithelial dysplasia. Clin Cancer Res 2000; 6:357; PMID:10690511.

[27] Mao L, Lee JS, Fan YH, Ro JY, Batsakis JG, Lippman S, Hittelman W, Hong WK. Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment. Nat Med 1996; 2:682-5; PMID:8640560; http://dx.doi.org/10.1038/nm0696-682.

[28] Califano J, van der Riet P, Westra W, Nawroz H, Clayman G, Piantadosi S, Corio R, Lee D, Greenberg B, Koch W, et al. Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res 1996 Jun 1; 56(11):2488-92.

[29] Brzoska PM, Levin NA, Fu KK, Kaplan MJ, Singer MI, Gray JW, Christman MF. Frequent novel DNA copy number increase in squamous cell head and neck tumors. Cancer Res 1995 Jul 15; 55 (14):3055-9.

[30] Hermsen M, Guervós MA, Meijer G, Baak J, van Diest P, Marcos CA, Sampedro A. New chromosomal regions with high-level amplifications in squamous cell carcinomas of the larynx and pharynx, identified by comparative genomic hybridization. J Pathol 2001 Jun; 194(2):177-82; http://dx.doi.org/10.1002/path.862.

[31] Callender T, el-Naggar AK, Lee MS, Frankenthaler R, Luna MA, Batsakis JG. PRAD-1 (CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma. Cancer 1994 Jul 1; 74(1):152-8; http://dx.doi.org/10.1002/1097-0142(19940701)74:1%3c152::AID-CNCR2820740124%3e3.0.CO;2-K.

[32] Ioachim E, Peschos D, Goussia A, Mittari E, Charalabopoulos K, Michael M, Salmas M, Vougiouklakis T, Assimakopoulos D, Agnantis NJ. Expression patterns of cyclins D1, E in laryngeal epithelial lesions: correlation with other cell cycle regulators (p53, pRb, Ki-67 and PCNA) and clinicopathological features. J Exp Clin Cancer Res 2004 Jun; 23(2):277-83.

[33] Hibi K, Trink B, Patturajan M, Westra WH, Caballero OL, Hill DE, Ratovitski EA, Jen J, Sidransky D. AIS is an oncogene amplified in squamous cell carcinoma. Proc Natl Acad Sci U S A 2000 May 9; 97 (10):5462-7; http://dx.doi.org/10.1073/pnas.97.10.5462.

[34] Van Dyke DL, Worsham MJ, Benninger MS, Krause CJ, Baker SR, Wolf GT, Drumheller T, Tilley BC, Carey TE. Recurrent cytogenetic abnormalities in squamous cell carcinomas of the head and neck region. Genes Chromosomes Cancer 1994 Mar; 9(3):192-206; http://dx.doi.org/10.1002/gcc.2870090308.

[35] Nawroz H, van der Riet P, Hruban RH, Koch W, Ruppert JM, Sidransky D. Allelotype of head and neck squamous cell carcinoma. Cancer Res 1994 Mar 1; 54(5):1152-5.

[36] Van der Riet P, Nawroz H, Hruban RH, Corio R, Tokino K, Koch W, Sidransky D. Frequent loss of chromosome 9p21-22 early in head and neck cancer progression. Cancer Res 1994 Mar 1; 54 (5):1156-8.

[37] Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS 3rd, Johnson BE, Skolnick MH. A cell cycle regulator potentially involved in genesis of many tumor types. Science 1994 Apr 15; 264(5157):436-40; http://dx.doi.org/10.1126/science.8153634.

[38] Okami K, Reed AL, Cairns P, Koch WM, Westra WH, Wehage S, Jen J, Sidransky D. Cyclin D1 amplification is independent of p16 inactivation in head and neck squamous cell carcinoma. Oncogene 1999 Jun 10; 18(23):3541-5; http://dx.doi.org/10.1038/sj.onc.1202837.

[39] Liggett WH Jr, Sewell DA, Rocco J, Ahrendt SA, Koch W, Sidransky D. p16 and p16 β are potent growth suppressors of head and neck squamous carcinoma cells in vitro. Cancer Res 1996 Sep 15; 56 (18):4119-23.

[40] Yokoyama J, Shiga K, Sasano H, Suzuki M, Takasaka T. Abnormalities and the implication of retinoblastoma locus and its protein product in head and neck cancers. Anticancer Res 1996 Mar-Apr; 16(2):641-4.

[41] Semczuk A, Marzec B, Roessner A, Jakowicki JA, Wojcierowski J, Schneider-Stock R. Loss of heterozygosity of the retinoblastoma gene is correlated with the altered pRb expression in human endometrial cancer. Virchows Arch. 2002 Dec; 441(6):577-83. Epub 2002 Sep 25; http://dx.doi.org/10.1007/s00428-002-0695-9.

[42] Xing EP, Yang GY, Wang LD, Shi ST, Yang CS. Loss of heterozygosity of the Rb gene correlates with pRb protein expression and associates with p53 alteration in human esophageal cancer. Clin Cancer Res 1999 May; 5(5):1231-40.

[43] Rafferty M, Walker C, Husband D, Helliwell T, Fenton J, Jones A. Retinoblastoma gene abnormalities in early laryngeal cancer. Eur Arch Otorhinolaryngol 2008 Jul; 265 Suppl 1:S83-7.

[44] Poeta ML, Manola J, Goldwasser MA, Forastiere A, Benoit N, Califano JA, Ridge JA, Goodwin J, Kenady D, Saunders J, et al. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med 2007 Dec 20; 357(25):2552-61; http://dx.doi.org/10.1056/NEJMoa073770.

[45] Bradford CR, Zhu S, Poore J, Fisher SG, Beals TF, Thoraval D, Hanash SM, Carey TE, Wolf GT. p53 mutation as a prognostic marker in advanced laryngeal carcinoma. Department of Veterans Affairs Laryngeal Cancer Cooperative Study Group. Arch Otolaryngol Head Neck Surg 1997 Jun; 123(6):605-9; http://dx.doi.org/10.1001/archotol.1997.01900060047008.

[46] de Vicente C, Gutierrez LMJ, Zapatera AH, Fresno Forcelledo MF, Hernández-Vallejo G, López Arranz JS. Prognostic significance of p53 expression in oral squamous cell carcinoma without neck noted metastases. Head Neck 2004; 26:22; PMID:14724903; http://dx.doi.org/10.1002/hed.10339.

[47] Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, Fakhry C, Xie TX, Zhang J, Wang J, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science 2011; 333:1154-7; PMID:21798897; http://dx.doi.org/10.1126/science.1206923.

[48] Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, Kryukov GV, Lawrence MS, Sougnez C, McKenna A, et al. The mutational landscape of head and neck squamous cell carcinoma. Science 2011; 333:1157-60; PMID:21798893; http://dx.doi.org/10.1126/science.1208130.

[49] Sun W, Gaykalova DA, Ochs MF, Mambo E, Arnaoutakis D, Liu Y, Loyo M, Agrawal N, Howard J, Li R, et al. Activation of the NOTCH pathway in head and neck cancer. Cancer Res 2014 Feb 15; 74(4):1091-104; http://dx.doi.org/10.1158/0008-5472.CAN-13-1259.

[50] Krikelis D, Kotoula V, Bobos M, Fountzilas E, Markou K, Karasmanis I, Angouridakis N, Vlachtsis K, Kalogeras KT, Nikolaou A, et al. Protein and mRNA expression of notch pathway components in operable tumors of patients with laryngeal cancer. Anticancer Res 2014 Nov; 34(11):6495-503.

[51] Velu TJ. Structure, function and transforming potential of the epidermal growth factor receptor. Mol Cell Endocrinol 1990 May 7; 70 (3):205-16; http://dx.doi.org/10.1016/0303-7207(90)90211-P.

[52] Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor α and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res 1993 Aug 1; 53(15):3579-84.

[53] Temam S, Kawaguchi H, El-Naggar AK, Jelinek J, Tang H, Liu DD, Lang W, Issa JP, Lee JJ, Mao L. Epidermal growth factor receptor copy number alterations correlate with poor clinical outcome in patients with head and neck squamous cancer. J Clin Oncol 2007; 25(16):2164-70; PMID:17538160; http://dx.doi.org/10.1200/JCO.2006.06.6605.

[54] Dittmann K, Mayer C, Rodemann HP. Nuclear EGFR as novel therapeutic target: insights into nuclear translocation and function. Strahlenther Onkol 2010; 186(1):1-6; PMID:20082181; http://dx.doi.org/10.1007/s00066-009-2026-4.

[55] Maurizi M, Almadori G, Ferradina G, Distefano M, Romanini ME, Cadoni G, Benedetti-Panici P, Paludetti G, Scambia G, Mancuso S. Prognostic significance of epidermal growth factor receptor in laryngeal squamous cell carcinoma. Br J Cancer 1996; 74:1253-7; PMID:8883413; http://dx.doi.org/10.1038/bjc.1996.525.

[56] Nijkamp MM, Span PN, Terhaard CH, Doornaert PA, Langendijk JA, van den Ende PL, de Jong M, van der Kogel AJ, Bussink J, Kaanders JH. Epidermal growth factor receptor expression in laryngeal cancer predicts the effect of hypoxia modification as an additive to accelerated radiotherapy in a randomised controlled trial. Eur J Cancer 2013 Oct; 49(15):3202-9; http://dx.doi.org/10.1016/j.ejca.2013.06.024.

[57] Burtness B, Goldwasser MA, Flood W, Mattar B, Forastiere AA. Eastern Cooperative Oncology Group. Phase III randomized trial of cisplatin plus placebo compared with cisplatin plus cetuximab in metastatic/recurrent head and neck cancer: an Eastern Cooperative Oncology Group study. J Clin Oncol 2005 Dec 1; 23(34):8646-54; http://dx.doi.org/10.1200/JCO.2005.02.4646.

[58] Sok JC, Coppelli FM, Thomas SM, Lango MN, Xi S, Hunt JL, Freilino ML, Graner MW, Wikstrand CJ, Bigner DD, et al. Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. Clin Cancer Res 2006 Sep 1; 12(17):5064-73; http://dx.doi.org/10.1158/1078-0432.CCR-06-0913.

[59] Bonner WM. gammaH2AX and cancer. Nature Reviews Cancer 2008; 8:957-67; PMID:19005492; http://dx.doi.org/10.1038/nrc2523.

[60] Guijarro MV, Leal JF, Fominaya J, Blanco-Aparicio C, Alonso S, Lleonart M, Castellvi J, Ruiz L, Ramon Y, Cajal S, et al. MAP17 overexpression is a common characteristic of carcinomas. Carcinogenesis 2007; 28(8):1646-52; PMID:17426052; http://dx.doi.org/10.1093/carcin/bgm083.

[61] Perez M, Praena-Fernandez JM, Felipe-Abrio B, Lopez-Garcia MA, Lucena-Cacace A, Garcia A, Lleonart M, Roncador G, Marin JJ, Carnero A. MAP17 and SGLT1 protein expression levels as prognostic markers for cervical tumor patient survival. PLoS One 2013; 8(2):e56169; PMID:23418532; http://dx.doi.org/10.1371/journal.pone.0056169.

[62] de Miguel-Luken MJ, Chaves-Conde M, de Miguel-Luken V, Muñoz-Galván S, López-Guerra JL, Mateos JC, Pachón J, Chinchón D, Suarez V, Carnero A. MAP17 (PDZKIP1) as a novel prognostic biomarker for laryngeal cancer. Oncotarget 2015 May 20; 6 (14):12625-36; http://dx.doi.org/10.18632/oncotarget.3470.

[63] de Miguel-Luken MJ, Chaves-Conde M, de Miguel-Luken V, Muñoz-Galván S, López-Guerra JL, Mateos JC, Pachón J, Chinchón D, Suarez V, Carnero A. Phosphorylation of H2AX as a novel predictive biomarker for laryngoesophageal dysfunction-free survival. submitted.

[64] Murray D, Rosenberg E. The importance of the ERCC1/ERCC4 [XPF] complex for hypoxic-cell radioresistance does not appear to derive from its participation in the nucleotide excision repair pathway. Mutat Res 1996; 364:217-26; PMID:8960133; http://dx.doi.org/10.1016/S0921-8777(96)00036-5.

[65] Jun HJ, Ahn MJ, Kim HS, Yi SY, Han J, Lee SK, Ahn YC, Jeong HS, Son YI, Baek JH, et al. ERCC1 expression as a predictive marker of squamous cell carcinoma of the head and neck treated with cisplatin-based concurrent chemoradiation. Br J Cancer 2008; 99:167-72; PMID:18594541; http://dx.doi.org/10.1038/sj.bjc.6604464.

[66] Lu B, Li J, Gao Q, Yu W, Yang Q, Li X. Laryngeal cancer risk and common single nucleotide polymorphisms in nucleotide excision repair pathway genes ERCC1, ERCC2, ERCC3, ERCC4, ERCC5 and XPA. Gene 2014 May 25; 542(1):64-8; http://dx.doi.org/10.1016/j.gene.2014.02.043.

[67] Santos CR, Rodríguez-Pinilla M, Vega FM, Rodríguez-Peralto JL, Blanco S, Sevilla A, Valbuena A, Hernández T, van Wijnen AJ, Li F, et al. VRK1 signaling pathway in the context of the proliferation phenotype in head and neck squamous cell carcinoma. Mol Cancer Res 2006; 4:177-85; PMID:16547155; http://dx.doi.org/10.1158/1541-7786.MCR-05-0212.

[68] Thomas SJ, Snowden JA, Zeidler MP, Danson SJ. The role of JAK/STAT signalling in the pathogenesis, prognosis and treatment of solid tumours. Br J Cancer 2015; 113:365-71; PMID:26151455; http://dx.doi.org/10.1038/bjc.2015.233.

[69] O'Shea JJ, Schwartz DM, Villarino AV, Gadina M, McInnes IB, Laurence A. The JAK-STAT pathway: impact on human disease and therapeutic intervention. Annu Rev Med 2015; 66:311-28; PMID:25587654; http://dx.doi.org/10.1146/annurev-med-051113-024537.

[70] Hosford SR, Miller TW. Clinical potential of novel therapeutic targets in breast cancer: CDK4/6, Src, JAK/STAT, PARP, HDAC, and PI3K/AKT/mTOR pathways. Pharmacogenomics Pers Med 2014; 7:203-15; PMID:25206307.

[71] Furqan M, Akinleye A, Mukhi N, Mittal V, Chen Y, Liu D. STAT inhibitors for cancer therapy. J Hematol Oncol 2013; 6:90; PMID:24308725; http://dx.doi.org/10.1186/1756-8722-6-90.

[72] Bournazou E, Bromberg J. Targeting the tumor microenvironment: JAK-STAT3 signaling. Jak-Stat 2013; 2:e23828; PMID:24058812; http://dx.doi.org/10.4161/jkst.23828.

[73] Turano C, Gaucci E, Grillo C, Chichiarelli S. ERp57/GRP58: a protein with multiple functions. Cell Mol Biol Lett 2011; 16:539-63; PMID:21837552; http://dx.doi.org/10.2478/s11658-011-0022-z.

[74] Choe MH, Min JW, Jeon HB, Cho DH, Oh JS, Lee HG, Hwang SG, An S, Han YH, Kim JS. ERp57 modulates STAT3 activity in radioresistant laryngeal cancer cells and serves as a prognostic marker for laryngeal cancer. Oncotarget 2015; 6:2654-66; PMID:25605256; http://dx.doi.org/10.18632/oncotarget.3042.

[75] Gargalionis AN, Basdra EK. Insights in microRNAs biology. Curr Top Med Chem 2013; 13: 1493-1502; PMID:23745801; http://dx.doi.org/10.2174/15680266113139990098.

[76] Weidhaas J. Using microRNAs to understand cancer biology. Lancet Oncol 2010; 11:106-7; PMID:20022811; http://dx.doi.org/10.1016/S1470-2045(09)70386-9.

[77] Yu X, Li Z. The role of microRNAs expression in laryngeal cancer. Oncotarget 2015; 6:23297-305; PMID:26079642; http://dx.doi.org/10.18632/oncotarget.4195.

[78] Olivieri F, Albertini MC, Orciani M, Ceka A, Cricca M, Procopio AD, Bonafe M. DNA damage response (DDR) and senescence: shuttled inflamma-miRNAs on the stage of inflamm-aging. Oncotarget 2015; 6:35509-21; PMID:26431329.

[79] Zhang SY, Lu ZM, Lin YF, Chen LS, Luo XN, Song XH, Chen SH, Wu YL. 2016. miR-144-3p, a tumor suppressive microRNA targeting ETS-1 in laryngeal squamous cell carcinoma. Oncotarget 7(10):11637-11650; PMID:26826553. doi:10.18632/oncotarget.7025.

[80] Chevalier D, Laccourreye O, Brasnu D, Laccourreye H, Piquet JJ. Crycohyoidoepiglottopexy for glottic carcinoma with fixation o impaired motion of the true vocal cord: 5-year oncology results with 112 patients. Ann Otol Rhinol Laryngol 1997; 106(5):364-69.

[81] Silver CE, Beitler JJ, Shaha AR, Rinaldo A, Ferlito A. Current trends in initial management of laryngeal cancer: the declining use of open surgery. Eur Arch Otorhinolaryngol 2009; 266(9):1333-52; PMID:19597837; http://dx.doi.org/10.1007/s00405-009-1028-2.

[82] Jørgensen K, Godballe C, Hansen O, Bastholt L. Cancer of the larynx: treatment results after primary radiotherapy with salvage surgery in a series of 1005 patients. Acta Oncol 2002; 41:69-76.

[83] American Society of Clinical Oncology, Pfister DG, Laurie SA, Weinstein GS, Mendenhall WM, Adelstein DJ, Ang KK, Clayman GL, Fisher SG, Forastiere AA, Harrison LB, et al. American Society of Clinical Oncology clinical practice guideline for the use of larynx-preservation strategies in the treatment of laryngeal cancer. J Clin Oncol 2006; 24:3693; http://dx.doi.org/10.1200/JCO.2006.07.4559.

[84] Forastiere AA, Goepfert H, Maor M, Pajak TF, Weber R, Morrison W, Glisson B, Trotti A, Ridge JA, Chao C, et al. Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. N Engl J Med 2003; 349(22):2091-8; PMID:14645636; http://dx.doi.org/10.1056/NEJMoa031317.

[85] Forastiere AA, Zhang Q, Weber RS, Maor MH, Goepfert H, Pajak TF, Morrison W, Glisson B, Trotti A, Ridge JA, et al. Long-term results of RTOG 91–11: a comparison of three nonsurgical treatment strategies to preserve the larynx in patients with locally advanced larynx cancer. J Clin Oncol 2013 Mar 1; 31(7):845-52; http://dx.doi.org/10.1200/JCO.2012.43.6097.

[86] Pignon JP, le Maitre A, Maillard E, Bourhis J for the MACH-NC Collaborative Group. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomized trials and 17,346 patients. Radiother Oncol 2009; 92(1): 4-14; PMID: 19446902; http://dx.doi.org/10.1016/j.radonc.2009.04.014.

[87] Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, Jones CU, Sur R, Raben D, Jassem J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med 2006 Feb 9; 354(6):567-78; http://dx.doi.org/10.1056/NEJMoa053422.

[88] Bonner JA, Harari PM, Giralt J, Baselga D, Shin R, Cohen J. Improved preservation of larynx with the addition of cetuximab to radiation for cancers of the larynx and hypopharynx. J Clin Oncol 2005 ASCO Annual Meeting Proceedings 2005; 23:5533.

[89] Pointreau Y, Garaud P, Chapet S, Sire C, Tuchais C, Tortochaux J, Faivre S, Guerrif S, Alfonsi M, Calais G. Randomized trial of induction chemotherapy with cisplatin and 5-fluorouracil with or without docetaxel for larynx preservation. J Natl Cancer Inst 2009; 101 (7):498-506; PMID:19318632; http://dx.doi.org/10.1093/jnci/djp007.

[90] Lefebvre JL, Pointreau Y, Rolland F, Alfonsi M, Baudoux A, Sire C, de Raucourt D, Malard O, Degardin M, Tuchais C, et al. Induction chemotherapy followed by either chemoradiotherapy or bioradiotherapy for larynx preservation: the TREMPLIN randomized phase II study. J Clin Oncol 2013; 31(7):853-9; PMID:23341517; http://dx.doi.org/10.1200/JCO.2012.42.3988.

[91] Ang K, Zhang Q, Wheeler RH, et al. A phase III trial (RTOG 0129) of two radiation-cisplatin regimens for head and neck carcinomas (HNC): Impact of radiation and cisplatin intensity on outcome. J Clin Oncol 2010; 28:422s (suppl; abstr 5507); http://dx.doi.org/10.1002/hed.24026.

[92] Ghi MG, Paccagnella A, Floriani I, Garavaglia D. Concomitant chemoradiation in locally advanced head and neck squamous cell carcinoma: A literature-based meta-analysis on the platinum concomitant chemotherapy. J Clin Oncol 2011; 29:2011-9 (suppl; abstr 5534); PMID:21502544.

[93] Strojan P, Vermorken JB, Beitler JJ, Saba NF, Haigentz M Jr, Bossi P, Worden FP, Langendijk JA, Eisbruch A, Mendenhall WM, et al. 2015. Cumulative cisplatin dose in concurrent chemoradiotherapy for head and neck cancer: A systematic review. Head Neck 2015; http://dx.doi.org/10.1002/hed.24026

[94] Vermorken JB, Specenier P. Optimal treatment for recurrent/metastatic head and neck cancer. Ann Oncol 2010 Oct; 21 Suppl 7: vii252-61.

[95] Argiris A, Li Y, Forastiere A. Prognostic factors and long-term survivorship in patients with recurrent or metastatic carcinoma of the head and neck. Cancer 2004 Nov 15; 101(10):2222-9; http://dx.doi.org/10.1002/cncr.20640.

[96] Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, Erfan J, Zabolotnyy D, Kienzer HR, Cupissol D, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med 2008; 359:1116-27; PMID:18784101; http://dx.doi.org/10.1056/NEJMoa0802656.

[97] Gibson MK, Li Y, Murphy B, Hussain MH, DeConti RC, Ensley J, Forastiere AA. Randomized phase III evaluation of cisplatin plus fluorouracil vs. cisplatin plus paclitaxel in advanced head and neck cancer (E1395): an intergroup trial of the Eastern Cooperative Oncology Group. J Clin Oncol 2005 May 20; 23(15):3562-7; http://dx.doi.org/10.1200/JCO.2005.01.057.

[98] Burtness B, Goldwasser MA, Flood W, Mattar B, Forastiere AA. Phase III randomized trial of cisplatin plus placebo compared with cisplatin plus cetuximab in metastatic/recurrent head and neck cancer: an Eastern Cooperative Oncology Group study. J Clin Oncol 2005 Dec 1; 23 (34):8646-54; http://dx.doi.org/10.1200/JCO.2005.02.4646.

[99] Grau JJ, Caballero M, Verger E, Monzó M, Blanch JL. Weekly paclitaxel for platin-resistant stage IV head and neck cancer patients. Acta Otolaryngol 2009 Nov; 129(11):1294-9; http://dx.doi.org/10.3109/00016480802590451.

[100] Machiels JP, Haddad RI, Fayette J, Licitra LF, Tahara M, Vermorken JB, Clement PM, Gauler T, Cupissol D, Grau JJ, et al. Afatinib versus methotrexate as second-line treatment in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck progressing on or after platinum-based therapy (LUX-Head & Neck 1): an open-label, randomised phase 3 trial. Lancet Oncol 2015 May; 16(5):583-94; http://dx.doi.org/10.1016/S1470-2045(15)70124-5.

[101] http://seer.cancer.gov.

[102] Fayette J, Guigay J, Le Tourneau C, Degardin M, Peyrade F, Orlandini F, Buffard K, Bellera C. Cabazitaxel in patients with refractory recurrent or metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN): Results of phase II trial unicancer ORL03. J Clin Oncol 32, 2014 (suppl; abstr e17028). Clinical trial information:NCT01620242.

MAP17 (PDZKIP1) as a novel prognostic biomarker for laryngeal cancer

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ABSTRACT

Larynx cancer organ preservation treatments with chemo and radiotherapy have substantially improved laryngoesophageal dysfunction-free survival. However, both of them lead to a high incidence of acute and chronic toxicities and a significant number of patients relapse. To date, there is no evidence available to establish the group of patients that may benefit from preservation approaches and clinical criteria such as primary tumor extension or pretreatment tracheotomy are not validated. MAP17 is a small non-glycosylated membrane protein overexpressed in carcinomas. The tumoral behavior induced by MAP17 is associated with reactive oxygen species production in which SGLT1 seems involved. In this study we found that the levels of MAP17 were related to clinical findings and survival in a cohort of 58 patients with larynx cancer. MAP17 expression is associated with overall survival (p<0.001) and laryngoesophageal dysfunction-free survival (p=0.002). Locoregional control in patients with high MAP17 showed better outcomes than those with low MAP17 (p=0.016). Besides, a positive correlation was observed between MAP17 expression and SGLT (p=0.022) and the combination of high levels of MAP17/SGLT also led to an increased overall survival (p=0,028). These findings suggest that MAP17, alone or in combination with SGLT1, may become a novel predictive biomarker for laryngeal carcinoma.

INTRODUCTION

Squamous cell carcinoma of the head and neck represents 4% of all cancers diagnosed worldwide, with >500.000 new cases recorded in 2008. Of them, 151.000 cases (130.000 men and 21.000 women) were laryngeal cancer with an estimated age-standardized world mortality rate of 2.3/100.000 habitants. In fact, 82.000 deaths were estimated in 2008 due to this cause [1].

Alcohol and tobacco abuse are common etiologic factors [2] but exposure to hard-alloys dust, chlorinated solvents [3] and familiar genetic patterns [4] have been also implicated. The role of Human Papilomavirus (HPV) is well established for squamous cell carcinoma of the oropharynx but it remains unclear for laryngeal cancer [5]. Besides, these patients are at risk of developing second primary tumors due to chronic aerodigestive tract carcinogen exposure: 14% in 5 years, 26% in 10 years and 37% in 15 years [6].

The main prognostic factor for overall survival (OS) is tumor staging, where node invasion is more relevant than tumor extension [7]. Other OS prognostic factors are

patient's comorbidity, performance status-ECOG (PS) [8], persistent toxic consumption habits [9], second primary tumor appearance [10] and primary tumor localization. In particular, glottic tumors reach 81% OS rates meanwhile supraglottic tumors drop to 70%, probably due to early detection [11]. Moreover, PS [8,12], node invasion [12,13] and localization [8] are prognostic factors for disease-free survival in conjunction with pathologic stage (pT) [13], surgical resection margins [12] and pretreatment tracheotomy [14].

Until the 1980s, total laryngectomy surgery was the standard treatment with subsequent loss of speech and airway patency [15]. Consequently, treatment aims changed in order to improve patient's quality of life through larynx sparing approaches. Organ preservation treatments for laryngeal cancer patients depend on whether the tumor is presented in early stages (I and II) or advanced locoregional disease (stage III/IV). In general, early stages are treated with either primary surgery or definitive radiotherapy (RT). Five-year OS in patients with stage I or stage II disease is typically 70 to 90%. Surgery and RT seem to have similar effectiveness in this setting although they have not been compared in a randomized trial [16,17]. Advanced disease requires multimodal approach, usually a combination of chemotherapy (CT) or biotherapy (B) with cetuximab plus RT. Although functional organ sparing approaches permit larynx preservation they do not provide a survival advantage over total laryngectomy [18]. Currently, three sparing approaches are accepted: RT, bio/chemo/radiotherapy (B/CTRT) and induction CT (ICT) followed by B/CTRT.

Regarding B/CTRT, cisplatin showed higher preservation rates as compared with induction CT followed by RT or RT alone (88% vs. 75% and 70%, respectively) with similar two and five year survival [19]. Later, a 10-year follow up publication confirmed that the arms that included CT improved laryngectomy-free survival [20]. Besides, a subsequent meta-analysis for locally advanced larynx cancer found that adding CT concomitant with RT led to a benefit of 6.5% absolute improvement in 5-year OS [21]. As for bio-radiotherapy, RT plus cetuximab showed better locoregional control rates than RT alone for advanced head and neck cancers [22]. A subset analysis of this study with hypopharyngeal and laryngeal carcinoma patients showed a hazard ratio (HR) 0.62 preservation in the cetuximab arm but this was not statistically significant [23].

With respect to ICT, the Veterans study demonstrated 64% preservation larynx rate without worsening survival in the induction followed by RT arm compared with surgery plus RT [24]. Afterwards, induction treatments were developed and the docetaxel, cisplatin and 5-Fluorouracil (DCF) schedule became the standard treatment, preserving the larynx 15% more than cisplatin and 5-Fluorouracil [25]. Furthermore, cetuximab has been studied as a concurrent treatment with RT instead of cisplatin after DCF induction chemotherapy, showing similar larynx preservation results in a phase II trial [26].

However, these strategies of treatment entail up to a 43% rate of late toxicities [27] and have not shown to prolong OS more than radical treatments. Interestingly, 5-year OS has decreased from 67.4% in 1985 to 59.6% in 2006 despite the development of new therapies for larynx cancer (Source: Surveillance, Epidemiology and End Results Program. Accessed: http://seer.cancer.gov/). One of the reasons that could justify this issue is that preservation approaches were not broadly used until the time of database collection. These two facts lead to the need for developing predictive biomarkers in order to select the patients that may benefit from preservation techniques and are not going to suffer unnecessary toxicities.

MAP17 is a small 17 Kda membrane protein present in a high proportion of tumors, not only in carcinoma. It has been found present in adenoma and benign tumors, and is highly expressed in metastatic carcinoma. Furthermore, its expression correlates with staging and malignant status of the tumor [28]. The expression is mainly driven at a transcriptional level either by promoter activation or demethylation [29, 30]. Expression of MAP17 in primary cells triggers senescence through p38, but in tumoral cells enhances the malignant capabilities of these cells, increasing proliferation, migration, resistance to apoptosis, etc [31,32]. MAP17 expression increases the levels of reactive oxygen species (ROS) in cells which may account for some of the increased tumoral properties [33]. In turn, a further increase of ROS might switch the balance towards apoptosis. Thus, MAP17 may increase the efficacy of therapies increasing ROS and therefore constitute a biomarker for better prognosis of these tumors. In cervix tumors treated with cisplatin and radiotherapy, high levels of MAP17 mark good survival of the patients [30]. Therefore, MAP17 is not only a marker for stage and malignant status but also may be a marker of prognosis and response to therapies involving oxidative stress. In this manuscript we have explored the relevance of the presence of MAP17 in larynx tumors where primary response is mainly achieved by treatments with radiotherapy and cisplatin or other radiosensitizers.

RESULTS

Clinical cohort description

At the time of the analysis, 21 (32%) deaths and 31 (48%) recurrences had occurred with a median follow-up of 29 months. The most common cause of LDS failure was local recurrence, which required salvage laryngectomy in 16 (52%) of cases. Mean OS was 58.2 months (48.7-67.7, CI 95%) and mean LDS 44.6 (35.2-54.1, CI 95%).

Lymph node metastases were significantly associated with decreased OS (N0: 63.1 m, N1: 38.6 and N2 22.2 m, p=0.019) while tumor local extension impacted LDS negatively (T4 extension 7.3 m vs. 47.1m non T4 extension, p=0.003). Besides, patients who required pretreatment tracheotomy had significantly worse LDS (54.3 vs. 18.9 months, p=0.001). The two-year cumulative proportion of patients with larynx preservation and OS were 57% and 76% respectively. Besides, locoregional control rate at two years was 60%. Therefore, our cohort behaves similarly to others reported in the literature [19,24].

MAP17 expression in larynx tumor samples

Out of 65 samples, only 58 were analyzed for MAP17 expression, either due to technical problems or because they did not contain any tumor cellularity. Out of the 58 samples, 46 (79%) were positives for MAP17 expression (Figure 1A,B and 1C) and there was a trend showing higher levels of MAP17 in advanced grades of the tumor (Figure 1D), although in this case, probably due to the low number of cases, it was not statistically significant. Surrounding normal tissue did not express MAP17 or expressed very low levels. We also analyzed other markers for proliferation such as KI67 or the activated form of ERK (phosphorylated ERK, ERK-p), or apoptosis such as mutant p53 or activated AKT (phosphorylated AKT, AKT-p). Our cohort showed a percentage of samples positive for KI67, mutant p53, ERK-p or AKT-p, but these groups did not show correlation with MAP17 levels (Figure 2). However, KI67 positivity showed statistically significant correlation with OS (Table 2). No correlation of MAP17 was observed with clinical parameters such as tumor localization, smoking habit, alcohol consumption, tumoral stage, pre-treatment tracheotomy and development of acute toxicities during chemoradiotherapy.

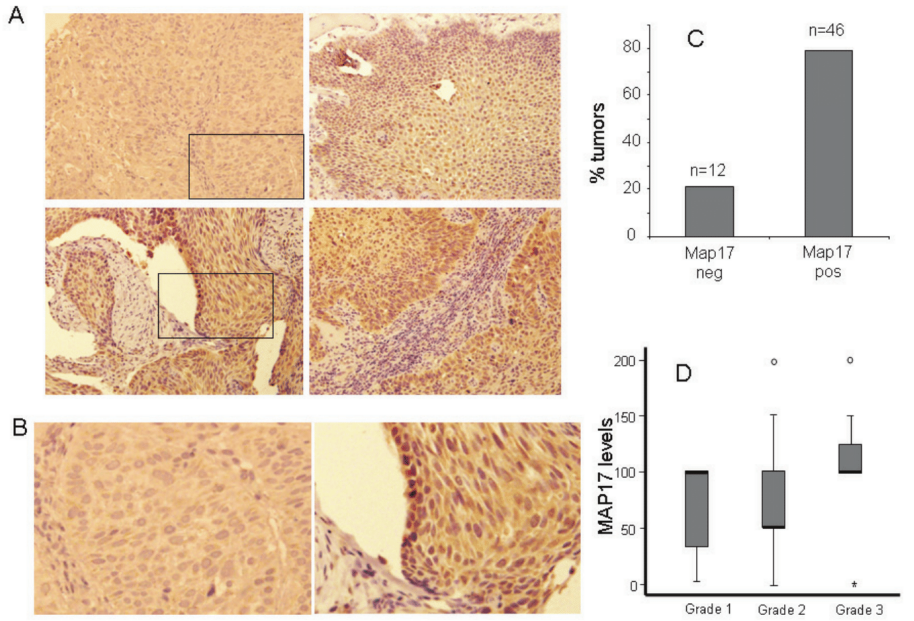

Figure 1: MAP17 overexpression in larynx tumors. A) Representative images of MAP17 immunostaining are shown for different larynx tumors. B) High magnification of M17 positive and negative tumors. The picture shows a magnification of the inset of figure A. C) A graph is shown representing the percentage of laryngeal tumors with dichotomous MAP17 levels. The score for positive tumors were >62. D) The distribution of the MAP17 expression levels among different grades of larynx tumors is shown. The MAP17 levels (score) refers to maximum levels (0–2) scored by the percentage of cells (0–100). The normalized levels were obtained by multiplying the percentage of cells by the level of intensity observed. Anova test was performed to establish the statistical association between MAP17 protein levels and the grade of the tumor ($p<0,05$).

Table1: Population characteristics and treatment.

Characteristics	No.	%
Mean age	62 years	
Male	61	94
Squamous cell carcinoma	65	100
Cigarette smoking Current smokers Former smokers Never smokers	 44 19 2	 68 29 3
Smokers of ≥ 10 pack-years	60	92
Regular alcohol intake	46	71
PS 0-1	62	95
Pretreatment tracheotomy	21	32
Localization Supraglottic Glottic Subglottic	 39 24 2	 60 37 3
TNM Staging II III IV	 6 49 10	 9 75.5 15.5
Treatment approach Surgery Radiotherapy B/CTRT ICT-B/CTRT	 1 9 49 6	 2 14 75 9

PS: Performance status-ECOG; B/CTRT: bio/chemoradiotherapy; ICT-B/CTRT: induction chemotherapy followed by bio/chemoradiotherapy.

Table 2: Laryngoesophageal dysfunction-free survival (LDS) and overall survival (OS) multivariate analysis of laryngeal cancer patients treated with preservation approaches.

	LDS			OS		
Factors	HR	p-value	CI	HR	p-value	CI
Ki67	1.001	0.938	0.976-1.027	1.053	0.050	1.000-1.108
P53	0.982	0.209	0.956-1.010	1.016	0.477	0.972-1.062
SGLT	1.007	0.238	0.995-1.019	0.993	0.497	0.972-1.014
MAP17	32.66	0.001	4.352-245.1	21.73	0.010	2.071-228.1
PS=2	13.99	0.019	1.534-127.7	4.162	0.446	0.106-162.8
Pret. Tracheo.	1.476	0.431	0.560-3.890	1.849	0.505	0.304-11.26
RT	0.161	0.258	0.007-3.804	0.042	0.161	0.000-3.549
Bio/CTRT	0.046	0.080	0.001-1.452	0.109	0.447	0.000-33.00
ICT-bio/CTRT	0.047	0.101	0.001-1.805	0.014	0.169	0.000-6.054
Stage II	0.224	0.202	0.023-2.228	0.402	0.703	0.004-43.73
Stage III	0.636	0.500	0.171-2.367	0.048	0.016	0.004-0.569

Pret. Tracheo.: pretreatment tracheotomy required. PS: ECOG-performance status. B/CTRT: bio/chemoradiotherapy; ICT-B/CTRT: induction chemotherapy followed by bio/chemoradiotherapy.

SGLT1 overexpression in human larynx tumors correlates with MAP17 levels

Previous results indicate that MAP17-dependent tumorigenic properties depend on the indirect activation of ROS by SGLT1 transport and that there is a correlation between the expressions of both markers in cervix tumors [30]. Therefore, we measured SGLT1 expression levels in the same cohort of larynx tumor samples. We found that some tumors showed positive SLGT1 staining, with approximately 40% tumors being positive for SLGT1 (Figure 3 A,B,C and D). However, only a few samples showed very high staining levels. The distribution of the SGLT1-positive tumors among the different larynx tumors showed a clear correlation with MAP17 expression (Figure 3E,F and G). Pearson indicator expressed a positive significant correlation between MAP17 and SGLT (P=0.3, p=0.022).

MAP17 as predictive biomarker for laryngeal cancer

The high MAP17 group correlated in this study with better OS, LDS and locoregional control. When MAP17 was measured as a constant variable, multivariate Cox model demonstrated that higher rates of MAP17 levels correlated with improved OS (HR: 0.98, p= 0.001). Nevertheless, this could not be confirmed for LDS (HR 0.99, p=0.8), probably due to the limited number of cases.

In order to distinguish a cutoff point for MAP17 levels a ROC curve was performed and punctuation of 62 score chosen. When measured as a dichotomous variable, MAP17 high rates (>62) were related with increased OS, LDS, and locoregional control. A difference of 35.3 months was observed between high MAP17 levels (67 months) and low MAP17 levels (31.7 m) in Kaplan-Meier model (IC 95%; p<0.001) and the HR estimator for high MAP17 was 0.78, p=0.002 in the multivariate analysis (Figure 4). Regarding LDS, high MAP17 showed a survival benefit of 13.1m (47.6 m vs. 34.5 m, p=0.002) with a HR: 0.14, p=0.003 in multivariate analysis. The effect of MAP17 high levels on the improved survival was significant after controlling for other variables: P53, Ki67, SGLT, PS, TNM, pretreatment tracheotomy and treatment received and shown in table 2. Regarding locoregional control, patients with high MAP17 showed to have better outcomes than low MAP17 (53.9 m vs 44.5 m, p=0.016) and the results were confirmed in the multivariate model (p=0.045).

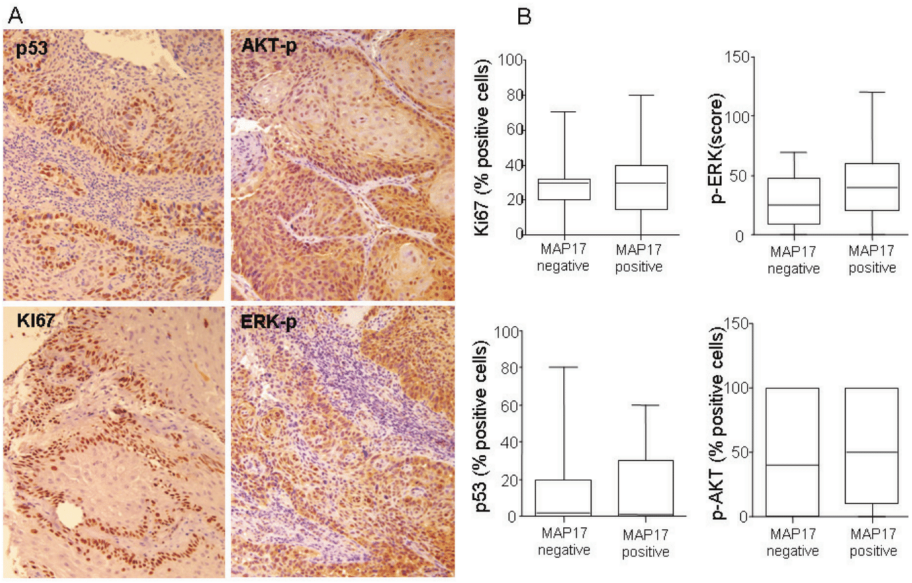

Figure 2: p53, Ki67, p-ERK or p-AKT do not show correlation with MAP17 expression in larynx tumors. A) Representative images of p53, Ki67, p-ERK or p-AKT immunostainings are shown for larynx tumors. B) Graphs showing lack of correlation between these proliferative or antiapoptotic markers and MAP17 expression.

However, although MAP17 correlated with SGLT in the Pearson model (Figure 3E), SGLT alone did not show statistically significant correlation with OS or LDS (results not shown). Moreover, the associated high levels of MAP17 and SGLT did show improved OS than MAP17 alone (72.4 m vs 42m, p=0,028) (Figure 4D).

These data confirm that MAP17 alone, or preferably combined with SGLT1, is a good prognostic marker for survival in patients with larynx cancer treated with B/CTRT.

Tumor cells overexpressing MAP17 are more sensitive to radiation

To explore whether MAP17 may be causal in this response, we expressed MAP17 cDNA in Hela cells and subjected these cells and their parental expressing only empty vector, to different doses of radiotherapy. Our data showed that Hela cells expressing MAP17 (Figure 5A) were more sensitive to radiation than parental cells without MAP17 (Figure 5B), therefore confirming the causal role of MAP17 in the sensitivity to radiation.

Finally, to test our initial hypothesis of the relevance of ROS in the MAP17-enhanced radiosensitivity of Hela cells, we treated the cells with antioxidants GSH and NAC, and subjected these cells to different radiation doses (Figure 5C and D). We observed that both antioxidant treatments reduced the sensitivity of MAP17-expressing Hela cells to a range similar to parental cells, which remains mostly unaltered (Figure 5C and D).

These data confirm the relevance of the oxidative status of the tumors in the response to radiation.

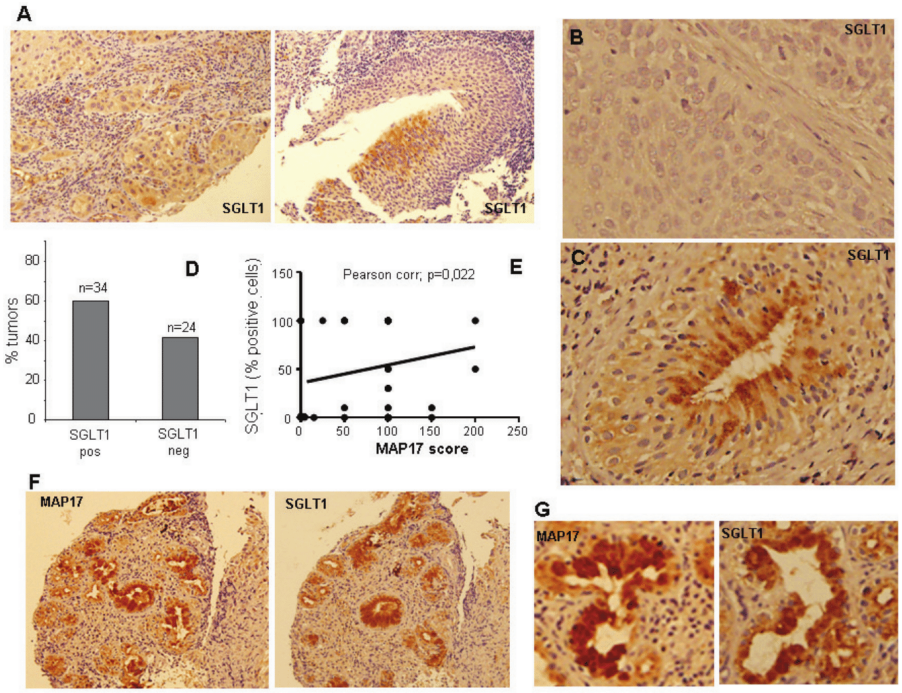

Figure 3: SGLT1 overexpression in larynx tumors. A) Representative images are shown of SGLT1 immunostaining of different larynx tumors. B) High magnification of SGLT1 positive and C) negative tumors. D) Graph representing the percentage of larynx tumors positive or negative for SGLT1 expression. E) Graph representing the correlation between MAP17 and SGLT1 expression in each tumor. The statistical analysis was performed by Pearson correlation (p=0,0022). F) Samples from one patient showing clear correlation between the expression of MAP17 and SGLT1. G) High magnification of samples from one patient showing clear correlation between the expression of MAP17 and SGLT1.

DISCUSSION

MAP17 is a small 17 kDa non-glycosylated membrane protein overexpressed in human carcinomas [29]. Tumor cells with MAP17 overexpression show enhanced proliferative capabilities [31,32]. MAP17 expression is associated with an SGLT-dependent ROS increase that acts as a second messenger enhancing tumorigenesis. While a mild increase in ROS has been shown to activate signaling cascades that upregulate tumorigenic processes, further ROS increases lead to a potentially toxic cellular environment and programmed cell death [33]. The hypothesis is that tumors expressing high levels of ROS producing MAP17 and SGLT1 proteins can benefit from therapies such as cisplatin or radiotherapy that increase oxidative stress and could sensitize them to cell death. In this setting, our analysis in laryngeal cancer showed a significant relationship between high MAP17 protein expression and increased OS, suggesting that MAP17 expression is an independent biomarker for survival. In fact, high MAP17 levels demonstrated better OS than low levels (67 months vs. 31.7 months, IC 95%; p<0.001). Our work also shows that patients with high MAP17 showed better locoregional control and LDS. Furthermore, the associated high levels of MAP17 and SGLT showed improved OS, better than MAP17 alone. These results are consistent with others presented in cervical cancer in which high levels of MAP17, better in

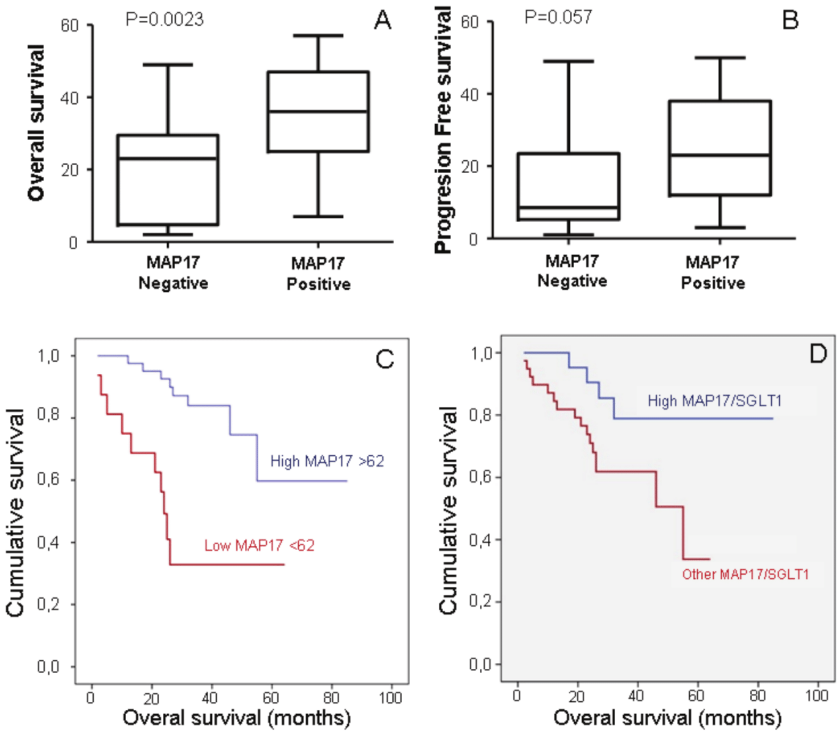

Figure 4: MAP17 alone or in combination with SGLT1 are good independent markers for patient survival. A) Correlation of MAP17expression measured as a dichotomous variable, MAP17 high rates (>62) with overall survival. B) Correlation of MAP17expression measured as a dichotomous variable, MAP17 high rates (>62) with laryngoesophageal dysfunction-free survival. C) A Kaplan-Meier curve is shown indicating that MAP17 could be a good prognostic marker for overall survival in laryngeal tumor patients treated with radiotherapy plus bio/chemotherapy. D) A Kaplan-Meier curve shown indicates that combined high levels of MAP17 and SGLT1 levels are a good prognostic marker for survival in cervical tumor patients treated with radiotherapy plus adjuvant chemotherapy.

combination with high SGLT1, correlated with improved patient survival after treatment [30]. Furthermore, proof of principle experiments *in vitro* demonstrated that antioxidant treatments reduced the sensitivity of MAP17-expressing Hela cells to a range similar to parental cells, confirming the relevance of the oxidative status of the tumors in the response to Radiation.

Our data confirm that MAP17 alone, and better in combination with SGLT1, is a good prognostic marker for survival in patients with larynx cancer treated with radiotherapy plus chemotherapy. Therefore, MAP17 could predict which patients may have better survival outcomes and would benefit from preservation approaches. Further prospective and controlled studies are needed in order to confirm our results and validate MAP17 as a novel biomarker of clinical use in larynx cancer.

METHODS

Patients characteristics and treatment

We evaluated 65 patients with larynx cancer and their treatment and evolution from August 2005 to February 2014. All patients expressed informed consent and the project was approved by the local ethical committee at HUVR. Patients received specific treatment in our institution while tumor samples were obtained from

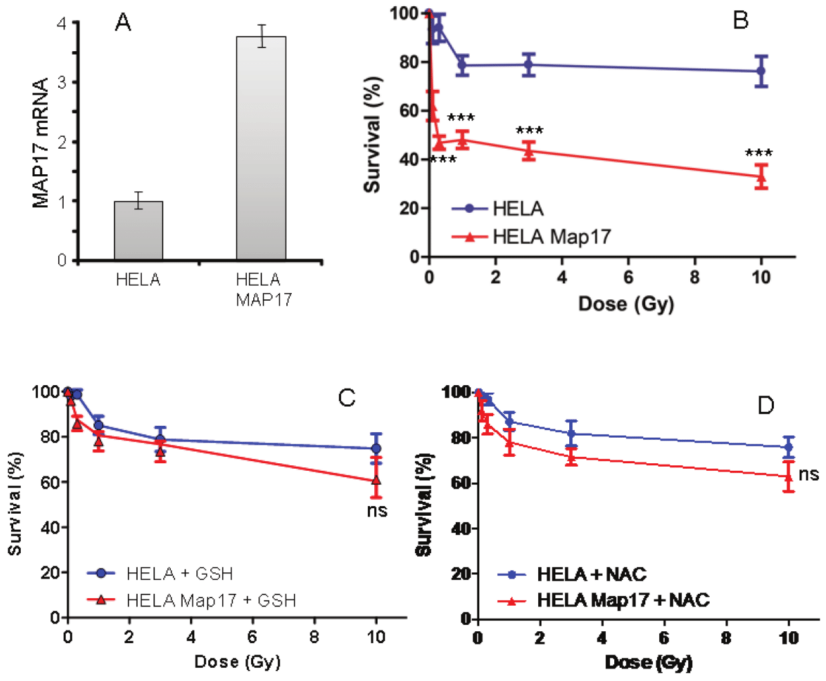

Figure 5: MAP17 overexpression in Hela cells induces sensitivity to radiotherapy. A) Hela cancer cells expressing ectopic MAP17 cDNA were selected and analyzed for MAP17 mRNA expression by quantitative RT-PCR. B) Hela cells expressing ectopically MAP17 cDNA (Hela-Map17) and parental cells expressing only empty vector (Hela) were seeded at equal concentration and subjected to different radiation doses as indicated, in triplicate samples. 48 hrs after treatment the percentage of survival cells was measured in each case and plotted in the graph. The experiment was performed three independent times in triplicate. C and D) Hela cells expressing ectopically MAP17 cDNA (Hela-Map17) and parental cells expressing only empty vector (Hela) were seeded at equal concentration and subjected to pretreatment with 10 mM GSH (C) or 10 mM NAC (D) during 18 hrs, then treated with different radiation doses as indicated, in triplicate samples. 48 hrs after treatment the percentage of survival cells was measured in each case and plotted in the graph.

four different national hospitals where the diagnosis was made. Eligibility criteria for preservation approaches in this study include patients with stage II-IV laryngeal tumors that had no contraindication for chemotherapy and/or radiotherapy, significant cartilage destruction, or more than 2 cm of tumor infiltration in the base of the tongue. TNM Staging System for the Larynx (7th ed., 2010) was used for tumor classification. Patients were mainly male (94%) with squamous carcinoma and good general condition (PS 0-1: 95%). Tumors were more frequently localized in the supraglottic (60%) and 75.5% were stage III. Interestingly, 32% of patients required pretreatment tracheotomy. Most of the patients were candidates for organ preservation with B/CTRT (75%), RT (14%), or ICT-B/CTRT (9%). Preferred treatment concurrent to radiotherapy was cisplatin 100 mg per square meter on days 1, 22, and 43 of radiotherapy (74%) followed by weekly cisplatin 40 mg per square meter (11%) and monoclonal antibody cetuximab (11%). Carboplatin was chosen for 4% of patients. Population characteristics and treatments are detailed in Table1.

Tissue acquirement and preparation.

Formalin-fixed, paraffin-embedded tissue sections from 65 laryngeal carcinomas were selected with the collaboration of the Andalusian Health Care Biological Resource Centre. Histological characterization of all samples was done by Hematoxylin and Eosin staining, followed by immunohistochemistry (IHQ) analysis of tissue microarrays (TMA).

Immunohistochemistry

Three-micrometer slices were sectioned from the TMA block and applied to coated, immunochemistry slides (DAKO, Glostrup, Denmark). The slides were baked overnight in a 56°C oven, deparaffinized in xylene for 20 min, rehydrated through a graded ethanol series and washed with PBS. A heat-induced epitope retrieval step was performed by heating a slide in a solution of sodium citrate buffer pH 6.5 for 2 min in a conventional pressure cooker. After heating, the slides were incubated with proteinase K for 10 min and rinsed in cool running water for 5 min. Endogenous peroxide activity was quenched with 1.5% hydrogen peroxide (DAKO) in methanol for 10 minutes, and incubation with the primary antibodies anti-MAP17 (1:4) [29-33] and anti-SGLT1 (Abcam #14685) was performed for 40 min. After incubation, immunodetection was performed with the EnVision (DAKO, Glostrup, Denmark) visualization system using diaminobenzidinechromogen as the substrate, according to the manufacturer's instructions. Immunostaining was performed in a TechMate 500 automatic immunostaining device (DAKO) and measured through a double-blind visual assessment using microscopic observation according to the anatomopathological experience of pathologists. Sample scoring was performed by semiquantitative microscopic analysis, considering the number of stained cells and signal intensity. Both MAP17 and SGLT stainings were performed. In both cases we used the score obtained by the multiplication of the intensity levels (1, 2 or 3) by the percentage of positive cells. For MAP17, the threshold used is the score of 62, obtained by ROC curve as the most relevant to establish as dichotomous variable. For SGLT1, there was no staining at all in non tumoral surrounding tissue, and the positivity was considered when positive cells were observed in the tumor.

Cell culture

Hela malignant cervical tumor cells were obtained from the European Collection of Cell Cultures (ECACC) human cell line repository and maintained in Dulbeccós modified Eaglés medium (Sigma) containing 10% fetal bovine serum (Sigma), penicillin, streptomycin and fungizone. MAP17 full-length cDNA was cloned into pBabepuro and mass culture generated by stable gene transfer in Hela cells. After selection with 2 µg/ML puromycin, mass cultures were used for the study. As a control, Hela cells were transfected with pBabepuro alone and selected.

Radiation treatment of *in vitro* culture

Cells were irradiated using Costar 24 well cell culture plates (Corning Incorporated, NY USA). To simulate actual radiobiological experimental conditions, each well was filled with culture medium. The plate dimensions were 12.5 x 8.5 cm. The inner diameter of the well was 16 mm and the distance between the centers of two neighboring wells was 20 mm. Plates were positioned inside a water-equivalent device, specifically designed to fit the plate. This device measures 16 x 16 x 2 cm, and is placed inside the IBA BodyPhantom (IBA Dosimetry GbmH, Schwarzenbruck, Germany) at a depth of 6 cm. Simulation was performed using a Toshiba Aquilion CT scanner (Toshiba Corporation, Japan). CT images were exported to the treatment planning system Philips Pinnacle V9.2 (Philips Radiation Oncology Systems, Madison, WI). Five plans were designed to deliver uniform doses of 0.1 Gray (Gy), 0.3 Gy, 1 Gy, 3 Gy and 10 Gy using static beams of 24 x18 cm. To verify the dose within every well, we delineated 24 regions of interest (ROI) that had a diameter of 16 mm. The ROI was estimated at the bottom of the wells and 5mm upwards. The dose delivered to the cells was verified with the IBA Compass system (IBA Dosimetry GbmH, Schwarzenbruck, Germany). Differences between the prescribed dose and the dose

received were within 3%. The irradiation was delivered using 6 megaelectronvolts (MV) photon beams from an Elekta Synergy Linac (Elekta Oncology System, Ltd, Crawley, UK) with a dose rate of 500 mu/min.

Statistical analysis and definitions

Kaplan-Meier method was used for survival analysis, using Cox Proportional Hazards model to adjust for the explanatory variables, obtain the p-values and estimate the HR. Multivariate logistic regression was used to obtain odds ratio (OR) and confidence intervals (CI 95%). Pearson's correlation measured dependence between quantitative variables. A receiver operating characteristic (ROC) curve was performed to assess MAP17 cutoff point (three year overall survival), which we checked using the optimal Youden index-based point. In addition, a log-rank test compared the survival distributions between the high MAP17 levels and the low levels. Statistical calculations were performed using SPSS 15.0 software.

OS has been defined as the length of time from the date of diagnosis until date of the last medical record. Adopting Lefebvre Larynx Preservation Consensus Panel (2009) for laryngoesophageal dysfunction-free survival (LDS), we considered endpoint events: death, local relapse, total or partial laryngectomy, tracheotomy at two or more years, or the presence of a feeding tube at two or more years [34]. Locoregional control was considered from the date of the diagnosis until local progression or last medical record, after excluding patients who developed distant metastases or died due to other causes not related to the larynx tumor.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

Disclaimer

Views expressed in the article are authors' own and not an official position of the institution or funders.

REFERENCES

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010; 127: 2893–2917.
2. Talamini R, Bosetti C, La Vecchia C, Dal Maso L, Levi F, Bidoli E, Negri E, Pasche C, Vaccarella S, Barzan L, Franceschi S. Combined effect of tobacco and alcohol on laryngeal cancer risk: a case-control study. Cancer Causes Control. 2002; 13(10):957-64.
3. Shangina O, Brennan P, Szeszenia-Dabrowska N, Mates D, Fabiánová E, Fletcher T, t'Mannetje A, Boffetta P, Zaridze D. Occupational exposure and laryngeal and hypopharyngeal cancer risk in central and eastern Europe. Am J Epidemiol. 2006; 164(4): 367-75.
4. Negri E, Boffetta P, Berthiller J, Castellsague X, Curado MP, Dal Maso L, Daudt AW, Fabianova E, Fernandez L, Wünsch-Filho V, Franceschi S, Hayes RB, Herrero R, et al. Family history of cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. Int J Cancer. 2009; 124(2): 394-401.
5. Torrente MC, Rodrigo JP, Haigentz M Jr, Dikkers FG, Rinaldo A, Takes RP, Olofsson J, Ferlito A. Human papillomavirus infections in laryngeal cancer. Head Neck. 2011; 33(4):581-6.
6. Gao X1, Fisher SG, Mohideen N, Emami B. Second primary cancers in patients with laryngeal cancer: a population-based study. Int J Radiat Oncol Biol Phys. 2003; 56:427-35.
7. Sessions D. Surgical pathology of cancer of the larynx and hypopharynx. Laryngoscope. 1976;132:504-7.
8. Singh B, Bhaya M, Stern J, Roland JT, Zimbler M, Rosenfeld RM, Har-El G, Lucente FE. Validation of the Charlson comorbidity index in patients with head and neck cancer: a multi-institutional study. Laryngoscope. 1997; 107(9 11 Pt1):1469-75.
9. Mayne ST, Cartmel B, Kirsh V, Goodwin Jr WJ. Alcohol and tobacco use prediagnosis and postdiagnosis, and survival in a cohort of patients with early stage cancers of the oral cavity, pharynx, and larynx. Cancer Epidemiol Biomarkers Prev. 2009; 18(12):3368-74.
10. Di Martino E, Sellhaus B, Hausmann R, Minkenberg R, Lohmann M, Esthofen MW. Survival in second primary malignances of patients with head and neck cancer. J Laryngol Otol. 2002; 116(10):831-8.

11. Raitiola H, Pukander J, Laippala P. Glottic and supraglottic laryngeal carcinoma: differences in epidemiology, clinical characteristics and prognosis. Acta Otolaryngol. 1999; 119(7):847-51.

12. Zhang SY, Lu ZM, Luo XN, Chen LS, Ge PJ, Song XH, Chen SH, Wu YL. Retrospective analysis of prognostic factors in 205 patients with laryngeal squamous cell carcinoma who underwent surgical treatment. PLoS One. 2013; 8(4):e60157.

13. Yilmaz T, Hoşal S, Ozyar E, Akyol F, Gürsel B. Post-operative radiotherapy in advanced laryngeal cancer: effect on local and regional recurrence, distant metastases and second primaries. J Laryngol Otol. 2005; 119(10):784-90.

14. Herchenhorn D, Dias FL, Ferreira CG, Araújo CM, Lima RA, Small IA, Kligerman J. Impact of previous tracheotomy as a prognostic factor in patients with locally advanced squamous cell carcinoma of the larynx submitted to concomitant chemotherapy and radiation. ORL J Otorhinolaryngol Relat Spec. 2008; 70(6):381-8.

15. Chevalier D, Laccourreye O, Brasnu D, Laccourreye H, Piquet JJ. Crycohyoidoepiglottopexy for glottic carcinoma with fixation o impaired motion of the true vocal cord: 5-year oncology results with 112 patients. Ann OtolRhinolLaryngol 1997; 106(5): 364-69.

16. Silver CE1, Beitler JJ, Shaha AR, Rinaldo A, Ferlito A. Current trends in initial management of laryngeal cancer: the declining use of open surgery. Eur Arch Otorhinolaryngol. 2009; 266(9):1333-52.

17. Jørgensen K, Godballe C, Hansen O, Bastholt L. Cancer of the larynx: treatment results after primary radiotherapy with salvage surgery in a series of 1005 patients. ActaOncol. 2002; 41:69-76.

18. Pfister DG, Laurie SA, Weinstein GS, Mendenhall WM, Adelstein DJ, Ang KK, Clayman GL, Fisher SG, Forastiere AA, Harrison LB, Lefebvre JL, Leupold N. American Society of Clinical Oncology clinical practice guideline for the use of larynx-preservation strategies in the treatment of laryngeal cancer. J Clin Oncol 2006; 24:3693.

19. Forastiere AA, Goepfert H, Maor M, Pajak TF, Weber R, Morrison W, Glisson B, Trotti A, Ridge JA, Chao C, Peters G, Lee DJ, Leaf A, et al. Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. N Engl J Med. 2003; 349(22):2091-2098.

20. Forastiere AA, Zhang Q, Weber RS, Maor MH, Goepfert H, Pajak TF, Morrison W, Glisson B, Trotti A, Ridge JA, Thorstad W, Wagner H, Ensley JF. Long-term results of RTOG 91-11: a comparison of three nonsurgical treatment strategies to preserve the larynx in patients with locally advanced larynx cancer. J Clin Oncol. 2013; 31(7):845-852.

21. Pignon JP, le Maître A, Maillard E, Bourhis J for the MACH-NC Collaborative Group. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomized trials and 17,346 patients. Radiotherapy and Oncology. 2009; 92(1): 4-14.

22. Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, Jones CU, Sur R, Raben D, Jassem J, Ove R, Kies MS, Baselga J. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med. 2006; 354(6):567-78.

23. Bonner JA, Harari PM, Giralt J, Baselga D, Shin R, Cohen J. Improved preservation of larynx with the addition of cetuximab to radiation for cancers of the larynx and hypopharynx. J Clin Oncol 2005 ASCO Annual Meeting Proceedings. 2005; 23:5533.

24. Wolf GT, Hong WK, Fisher SG, Urba S, Endicott JW, Close L, Toohill RJ, Karp D, Miller DM, Cheung NK, Weaver A, Hillel AD, Spaulding M. Induction chemotherapy plus radiation compared with surgery plus radiation in patients with advanced laryngeal cancer. The Department of Veterans Affairs Laryngeal Cancer Study Group. N Eng J Med. 1991; 324:1685-90.

25. Pointreau Y, Garaud P, Chapet S, Sire C, Tuchais C, Tortochaux J, Faivre S, Guerrif S, Alfonsi M, Calais G. Randomized trial of induction chemotherapy with cisplatin and 5-fluorouracil with or without docetaxel for larynx preservation. J Natl Cancer Inst. 2009; 101(7):498-506.

26. Lefebvre JL, Pointreau Y, Rolland F, Alfonsi M, Baudoux A, Sire C, de Raucourt D, Malard O, Degardin M, Tuchais C, Blot E, Rives M, Reyt E, et al. Induction chemotherapy followed by either chemoradiotherapy or bioradiotherapy for larynx preservation: the TREMPLIN randomized phase II study. J Clin Oncol. 2013; 31(7):853-9.

27. Machtay M, Moughan J, Trotti A, Garden AS, Weber RS, Cooper JS, Forastiere A, Ang KK. Factors associated with severe late toxicity after concurrent chemoradiation for locally advanced head and neck cancer: an RTOG analysis. J Clin Oncol. 2008; 26(21):3582-9.

28. Carnero A. MAP17 and the double-edged sword of ROS. Biochim Biophys Acta. 2012; 1826(1):44-52.

29. Guijarro MV, Leal JF, Fominaya J, Blanco-Aparicio C, Alonso S, Lleonart M, Castellvi J, Ruiz L, Ramon Y Cajal S, Carnero A. MAP17 overexpression is a common characteristic of carcinomas. Carcinogenesis. 2007; 28(8):1646-52.

30. Perez M, Praena-Fernandez JM, Felipe-Abrio B, Lopez-Garcia MA, Lucena-Cacace A, Garcia A, Lleonart M, Roncador G, Marin JJ, Carnero A. MAP17 and SGLT1 protein expression levels as prognostic markers for cervical tumor patient survival. PLoS One. 2013; 8(2):e56169.

31. Guijarro MV, Link W, Rosado A, Leal JF, Carnero A. MAP17 inhibits Myc-induced apoptosis through PI3K/AKT pathway activation. Carcinogenesis. 2007; 28(12):2443-50.

32. Guijarro MV, Vergel M, Marin JJ, Muñoz-Galván S, Ferrer I, Ramon y Cajal S, Roncador G, Blanco-Aparicio C, Carnero A. p38α limits the contribution of MAP17 to cancer progression in breast tumors. Oncogene. 2012; 31(41):4447-59.

33. Guijarro MV, Leal JF, Blanco-Aparicio C, Alonso S,

Fominaya J, Lleonart M, Castellvi J, Ramon y Cajal S, Carnero A. MAP17 enhances the malignant behavior of tumor cells through ROS increase. Carcinogenesis. 2007; 28(10):2096-104.

34. Lefebvre JL, Ang KK, Larynx Preservation Consensus Panel. Larynx preservation clinical trial design: key issues and recommendations –a consensus panel summary. Int J Radiat Oncol Biol Phys. 2009; 73(5):1293-303.

Phosphorylation of gH2AX as a novel prognostic biomarker for laryngoesophageal dysfunction-free survival

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ABSTRACT

Current larynx preservation treatments have achieved an improvement of laryngoesophageal dysfunction-free survival (LDS) but lead to significant toxicities and recurrences. At present, there is no evidence to select the group of patients that may benefit from preservation approaches instead of surgery. Therefore, laryngeal biomarkers could facilitate pretreatment identification of patients who could respond to chemoradiation-based therapy. In this study, we evaluated retrospectively 53 patients with larynx cancer to determine whether gH2AX phosphorylation (pH2AX) alone or in combination with the membrane protein MAP17 (PDZK1IP1) could be used as prognostic biomarkers. We also evaluated whether the completion of cisplatin treatment and radiotherapy could predict survival in combination with pH2AX.

We found that the dose of cisplatin received but not the length of the radiotherapy influenced LDS. High-pH2AX expression was associated with prolonged LDS (HR 0.26, *p* = 0.02) while MAP17 correlated with overall survival (OS) (HR 0.98, *p* = 0.05). High-MAP17 and high-pH2AX combined analysis showed improved LDS (with 61.35 months *vs* 32.2 months, *p* = 0.05) and OS (with 66.6 months *vs* 39.8 months, *p* = 0.01). Furthermore, the subgroup of high-pH2AX and optimal dose of cisplatin was also associated with OS (72 months *vs* 38.6 months, *p* = 0.03) and LDS (66.9 months *vs* 27 months, *p* = 0.017). These findings suggest that pH2AX alone or better in combination with MAP17 may become a novel and valuable prognostic biomarker for patients with laryngeal carcinoma treated with preservation approaches.

INTRODUCTION

Squamous cell carcinoma of the head and neck represents 4% of all cancers diagnosed worldwide, with more than 500.000 new cases recorded in 2008 [1, 2]. Of them, 30% were laryngeal cancer with an estimated age-standardized world mortality rate of 2.3/100.000 habitants. Alcohol and tobacco abuse are common etiologic factors [3] but exposure to hard-alloys dust, chlorinated solvents [4] and familiar genetic patterns [5] have been also implicated. The role of Human Papilomavirus (HPV) is well established for squamous cell carcinoma of the oropharynx but it remains unclear for laryngeal cancer [6]. Furthermore, these patients are at risk of developing second primary tumors due to chronic aerodigestive tract carcinogen exposure: 14% in 5 years, 26% in 10 years and

37% in 15 years [7]. The main prognostic factor for overall survival (OS) is tumor staging, where node invasion is more relevant than tumor extension [8]. Other OS prognostic factors are patient's comorbidity, performance status-ECOG (PS) [9], persistent toxic consumption habits [10], second primary tumor appearance [11] and primary tumor localization. In particular, glottic tumors have an 81% OS rate while supraglottic tumors drop to 70%, probably due to early detection [12]. Disease-free survival prognostic factors include PS [9, 13], node invasion [13, 14], localization [9], pathologic stage (pT) [14], surgical resection margins [13] and pretreatment tracheotomy [15]. Moreover, T4 primary extension and more than 2 cm tumoral invasion of the base of the tongue were shown to be associated with increased salvage laryngectomy in the Veterans study [16].

Total laryngectomy was the gold standard treatment by the 1980s with subsequent loss of speech and airway patency [17]. Consequently, treatment aims changed in order to improve patient's quality of life through larynx sparing approaches. Currently, early stages (I and II) are treated with either surgery or radiotherapy (RT) as they have been accepted to have similar effectiveness. However, both treatments have not been compared in a randomized trial so far. Reported five-year OS is typically 70 to 90% [18, 19]. Advanced disease requires multimodal approach, usually a combination of chemotherapy (CT) or biotherapy (B) plus RT. Although functional organ sparing approaches permit larynx preservation, they do not provide a survival advantage over total laryngectomy [20]. Three sparing approaches are accepted: RT, bio or chemotherapy with concomitant radiotherapy (B/CT RT) and induction CT (ICT) followed by RT with or without B/CT.

CTRT with concurrent cisplatin showed higher preservation rates compared to other two arms with RT alone or induction cisplatin plus fluorouracil followed by RT (88% *versus* -*vs*- 70% and 75%, respectively) with similar two and five year survival [21]. Later, a 10-year follow-up publication confirmed that the arms that included ICT improved laryngectomy-free survival (LFS). Contrary to preservation rates, LFS includes not just the need of salvage laryngectomy but also speech and swallowing quality. It is, therefore, more similar to what we currently understand as larynx preservation [22]. A subsequent meta-analysis for locally advanced larynx cancer found that adding CT concomitant with RT led to a benefit of 6.5% absolute improvement in 5-year OS [23].

The optimal dose of cisplatin during RT remains still unclear [24-26]. Two or three courses of three-weekly cisplatin could be considered the optimal dose for concurrent CTRT and equivalent doses of carboplatin have also been accepted by expert panels. However, preservation approaches entail up to a 43% rate of late toxicities [27] and have not shown to prolong OS more than surgery. Interestingly, 5-year OS was reported to drop from 67.4% in 1985 to 61.9% in 2007 (Source: Surveillance, Epidemiology and End Results Program. Accessed: http://seer.cancer.gov/). Nevertheless, these results do not allow making major conclusions, as preservation approaches were not broadly used until the time of database collection.

The high rate of toxicities and the non-improved survival with preservation approaches lead to the need for biomarker development. Predictive larynx biomarkers would facilitate pretreatment identification of those patients who are unlikely going to be cured by radiation-based therapy. By managing these patients with surgery rather than a preservation approach, local disease control and possibly survival could potentially be optimized and unnecessary treatment related morbidities from unsuccessful larynx treatments could be avoided. However, there are still no clinical or molecular biomarkers validated in standard practice at present.

γH2AX is a component of the histone octamer in nucleosomes. It is phosphorylated (pH2AX) by kinases such as ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) upon DNA damage. PH2AX is involved in recruiting DNA repair proteins in response to the presence of DNA double-strand breaks (DSB) and therefore it has been studied as a biomarker of DNA damage for new drug development. As such, the presence and magnitude of pH2AX is an indication of persistent, unrepaired DNA damage [28]. pH2AX induction appears within minutes in cells after DNA damage and reaches maximum levels after 30 minutes. The repair process includes the phosphorylation of hundreds to thousands γH2AX surrounding the DSB site in order to form a focus that open the chromatin structure and serve as a platform for the accumulation of factors involved in the DNA damage response [29]. γH2AX phosphorylation has been studied as prognostic biomarker in early operable non-small cell lung cancer (NSCLC) and endometrial carcinomas. In the NSCLC study, low levels of phosphorylated γH2AX correlated with better survival outcomes. The combination of wild type p53 and low-phosphorylated γH2AX phenotype showed also better survival. In the endometrial trial, p-γH2AX positively correlated with p53 levels although the relation with survival could not be proved. However, in both studies patients were treated with surgery and not with radiotherapy [30, 31].

Our goal was to determine whether pH2AX by itself or in combination with other molecular and clinical findings could be a prognosis biomarker for laryngeal carcinomas treated with RT alone or CTRT.

RESULTS

Clinical cohort description

Patients were mainly male (93.7%) with squamous carcinoma histopathology (100%) and good general condition (PS 0-1 = 96.8%). Tumors were more frequently localized in the supraglottic (58.7%) and 74.6% were stage III. Nodal involvement was observed in 25.4% of the patients and 6.3% had primary T4 extension. Pretreatment tracheotomy was required for 31.7% patients. Organ preservation approaches include B or CTRT (74.6%), RT (14.3%), or ICT-B/CTRT (9.5%) (Table 1). Most of the patients received concurrent CT or B during RT (82.5%). The preferred treatment was cisplatin 100 mg/m2 on days 1, 22, and 43 of radiotherapy (73%) followed by weekly cisplatin 40 mg/ m2 (11.5%) and cetuximab (11.5%). Carboplatin was selected for only 4% of patients.

At the time of the analysis, 20 (32%) deaths and 29 (46%) recurrences had occurred with a median follow-up of 29 months (m). Locoregional relapse occurred in 19 (30%) patients, 7 (11%) presented locoregional plus distant metastases, and 3 (4.8%) only distant metastases; of them, 14 (48.3%) were candidates for salvage surgery. Laryngoesophageal dysfunction (LD) occurred in 51% of the total; main reasons for LD were tumoral local recurrence (75%) followed by the need of a tracheostomy of feeding tube (15.6%). Mean OS was 58 m (47.7-68 m, CI 95%), LDS 46 m (36-55.5m, CI 95%), and LRC 54.6 m (44-65 m, CI 95%). Moreover, 2-year LRC rate was 63%. The 2-year cumulative proportion of patients with larynx preservation and OS were 57% and 80% respectively. Lymph node involvement was associated with worse OS (N0: 64.2m *vs* N1/2: 26.8 m, $p < 0.01$) but not with LDS (46.8 *vs* 24.6, $p = 0.6$) (Figure 1B and 1A). Tumor local extension impacted negatively on both OS and LDS (OS non-T4 60.7 m *vs* T4 22.5 m; LDS non-T4 48.5 m *vs* T4 7.3m, both $p = .001$) (Figure 1D and 1C). Furthermore, patients who required pretreatment tracheotomy (PT) had worse OS (37.2 m *vs* 61.8 m, $p = 0.051$) and LDS (19.6 m *vs* 55.4m, $p = 0.001$) (Figure 1F and 1E). Therefore, our cohort behaves similarly to others reported in the literature [16, 21].

Cisplatin and radiotherapy as prognostic markers in larynx cancer

As per the RTOG 0129 phase III clinical trial results, patient were classified by the dose of cisplatin received during the radiation treatment. In total, 52.4% reached the optimal dose of cisplatin during radiotherapy whereas 17.5% could not reach the cisplatin optimal dose, 12.7% did not receive any radiosensitizer, and 14.3% were treated with other radiosensitizers. 3.1% were unknown.

Cisplatin optimal dose (≥200 mg/m2) was associated with better outcomes for survival although this was statistically significant for LDS (OS: 67 m *vs* 39 m, $p = 0.073$; LDS: 56m *vs* 24 m, $p = 0.017$; LRC: 60.4 m *vs* 29 m, $p = 0.12$) (Figure 2B and 2A). Receiving an optimal dose of cisplatin showed better LDS ($p = 0.023$) than lesser doses (HR = 0.24), other radiosensitizers (HR = 0.32), and no concurrent radiosensitizers (HR = 0.65). However, this benefit was not observed for OS probably because the analysis did not take into account patients that needed salvage laryngectomy and did not preserve the organ.

On the other hand, total dose of radiation delivered was 70 Gy as per standard local guidelines. Patients that completed radiotherapy within 8 or 9 weeks were compared to those that suffered interruptions or delays but no differences were found in terms of OS or LDS between groups (Figure 3).

pH2AX in larynx tumor samples

Out of 63 samples only 53 were analyzed for pH2AX expression either due to technical problems or because they did not contain any tumor cellularity. Positive pH2AX expression, considered as any percentage of tumoral nuclei with positive staining, was shown in 46 (86.8%) samples with a range of 1 to 70 and median expression of 10 (Figure 4A). In order to distinguish a cutoff point for pH2AX levels a ROC curve was performed and punctuation of 5.25 score chosen (supplementary Figure 1). Levels of pH2AX were equally distributed among tumor grades (Figure 4B) suggesting independence from this clinical feature, as the Chi-square test showed no differences between groups ($p = 0.8$).

When measured as a continuous variable, pH2AX had a significant positive influence with better LDS outcomes (HR 0.95, $p = 0.02$), although this was not significant for OS. As a dichotomous variable, a trend towards better OS, LDS and LRC was observed but just LDS was statistically significant in the multivariate analysis (HR 0.26, $p = 0.02$) (Table 2) (Figure 3C, 3D and 3E).

pH2AX and clinical findings

We also studied the potential correlation between pH2AX and clinical findings such as tumor localization, tumoral stage, smoking habit, alcohol consumption and acute toxicity development with no statistically significant association. These results suggest pH2AX to be an independent prognostic factor, as it remains significant after controlling for these variables.

Table 1: Population characteristics and treatment

Population characteristics	No.	%
Mean age	63.7 years	
Male	59	93.7
Squamous cell carcinoma	63	100.0
Smokers of ≥ 10 pack-years	59	93.7
Regular alcohol intake	45	71.4
PS 0-1	61	96.8
Pretreatment tracheotomy	20	31.7
Cigarette smoking		
Current smokers	43	68.3
Former smokers	18	28.6
Never smokers	2	3.2
Localization		
Supraglottic	37	58.7
Glottic	24	38.1
Subglottic	2	3.2
TNM Staging		
II	6	9.5
III	47	74.6
IV	10	15.9
Treatment approach		
Surgery	1	1.6
Radiotherapy	9	14.3
B/CTRT	47	74.6
ICT-B/CTRT	6	9.5

PS: Performance status-ECOG; B/CTRT: bio/chemoradiotherapy; ICT-B/CTRT: induction chemotherapy followed by bio/chemoradiotherapy.

pH2AX relationship with cisplatin and radiotherapy

The total dose of cisplatin was not associated with pH2AX levels ($p = 0.4$). We created a variable with two categories from pH2AX and cisplatin, in which one had a potential favorable prognosis (high-pH2AX levels, and optimal dose of cisplatin, ≥200 mg/m2), and the other unfavorable prognosis (low-pH2AX levels, and/or suboptimal dose of cisplatin < 200mg/m2 or other radiosensitizers due to the low number of patients). The favorable prognosis group correlated with increased OS, LDS (OS: 72 m *vs* 38.6 m, $p = 0.03$; LDS 66.9 m *vs* 27 m, $p = 0.019$). LRC was not statistically significant ($p = 0.17$) although there was a trend towards better outcomes in the good prognostic subgroup (69.9 m *vs* 35.1 m) (Figure 5A, 5B and 5C). Moreover, the unfavorable prognosis group correlated with worse OS (HR = 3.66, $p = 0.044$), and LDS (HR = 3.38, $p = 0.028$). LRC has a not statistically significant HR (HR = 2.4, $p = 0.188$).

We also tried to stablish whether high-pH2AX and no radiotherapy delays could impact on survival but no differences were found for both OS and LDS.

Correlation of pH2AX with p53 and KI67

We also analyzed other markers for proliferation such as KI67 or the activated form of ERK (phosphorylated ERK, ERK-p), and apoptosis such as mutant (m) p53 or activated AKT (phosphorylated AKT, AKT-p). Our cohort showed a percentage of positive samples for ERK-p or AKT-p, but these groups did not show correlation with pH2AX expression (data not shown). KI67 in combination with pH2AX was not significant in any combination (data not shown), being pH2AX also independent of the proliferative capability of the tumor. Our results showed no correlation between p53 and pH2AX although there was a relation towards increased pH2AX with negative P53 (< 5% positive nuclei) that was not statistically significant ($p = 0.33$).

However, in our cohort p53 samples positive (measured as >5% positive nuclei) (Figure 6A, + p53) correlated with worse OS (- p53 = 50 *vs* + p53 = 35.6 m, $p = 0.047$) (Figure 6B) consistent with previous literature [34].

P53 and pH2AX were combined into a new variable with the following categories: potential good

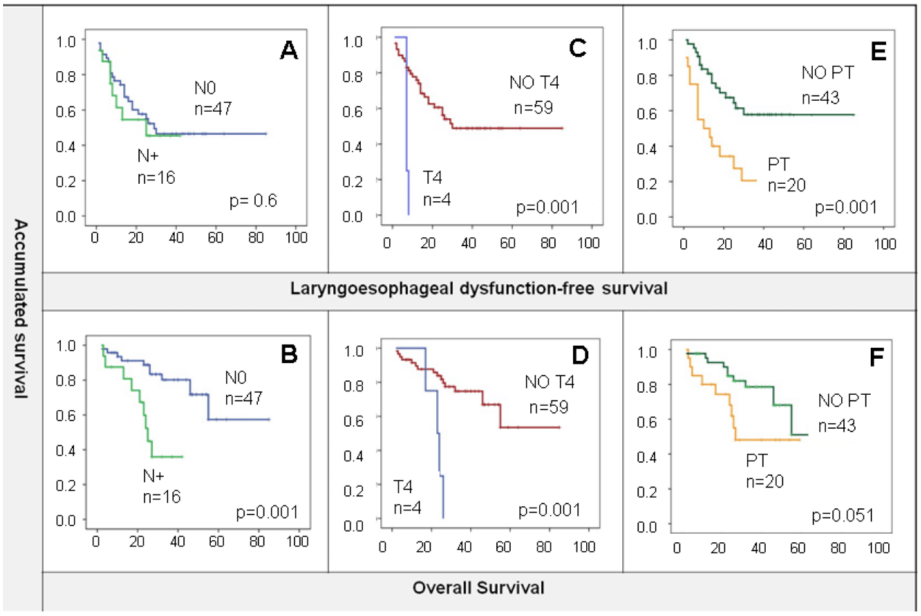

Figure 1: A. and **B.** N0 compared with N positive LDS/OS. OS was better in the patients who had no lymph node involvement. **C. and D.** T4 local tumor extension shows worse OS and LDS than the rest of patients. **E. and F.** worse LDS and OS is observed in patients who required pretreatment tracheotomy (PT).

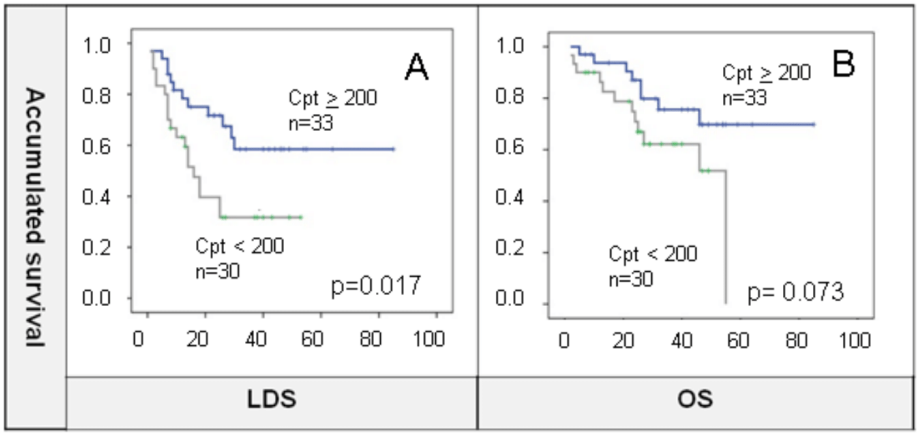

Figure 2: A. and **B.** Cisplatin (Cpt) optimal dose (≥200 mg/m2) showed significant LDS benefit that was not maintained for OS.

Table 2: LDS and OS multivariate analysis

LDS				OS			
	HR	***p*-value**	**CI**		**HR**	***p*-value**	**CI**
PT	1.41	0.59	0.39-5.08	**PT**	0.96	0.98	0.17-5.58
Non-primary T4 extension	0.09	0.00	0.02-0.49	**N negative**	0.65	0.03	0.01-0-74
Cisplatin >200mg/m²	0.09	0.00	0.02-0.43	**Cisplatin >200mg/m²**	0.33	0.29	0.04-2.56
High-pH2AX	0.26	0.02	0.09-0.78	**High-pH2AX**	0.57	0.46	0.13-2.55
KI 67	1.03	0.15	0.99-1.06	**KI 67**	1.06	0.038	1.00-1.12
MAP17	1.01	0.63	0.99-1.02	**MAP17**	0.98	0.05	0.95-1.00
P53	0.99	0.37	0.56-1.02	**P53**	1.03	0.12	0.99-1.07
pERK	0.99	0.35	0.97-1.01	**pERK**	1.01	0.65	0.98-1.04
pAKTP	0.99	0.41	0.98-1.01	**pAKT**	0.98	0.16	0.96-1.01

LDS: laryngoesophageal dysfunction-free survival. OS: overall survival. HR: hazard ratio. CI: confidence interval. PT: pretreatment tracheotomy. N: pathological lymph nodes.

prognosis phenotype (negative p53 and high-pH2AX) and unfavorable prognosis phenotype (positive p53 and low-pH2AX). Although there was an apparent relation towards better outcomes in the good prognosis phenotype, this was not significant for both OS and LDS (OS: 48.6 m *vs* 39 m, $p = 0.39$; LDS: 38.8 m *vs* 24.4 m, $p = 0.068$) (Figure 6C and 6D).

Correlation of pH2AX and MAP17

We have recently shown that MAP17, a small non-glycosylated membrane protein overexpressed in carcinomas, expression analyzed by immunohistochemistry is associated with OS ($p < 0.001$) and LDS ($p = 0.002$) [32]. MAP17 increases endogenous ROS [35, 36]. Since ROS is a well-known mediator of DNA damage [37], we measured whether pH2AX correlated with MAP17 expression and whether the combination of both markers could strength the predictability of responses.

We found that patients with high levels of MAP17 and subject to optimal doses of cisplatin had better LDS (58.6 m *vs* 32.6 m, $p = 0.053$) and OS (76.2 m *vs* 40.9 m, $p = 0.005$) than patients with low MAP17 or not subject to optimal doses of cisplatin (Figure 7A and 7B). Furthermore, patients with high levels of MAP17 and high-pH2AX, denoting higher structural DNA-damage, conform the group of better prognosis after therapy (Figure 7C and 7D).

Moreover, patients with high-MAP17, high-pH2AX and optimal dose of cisplatin had better OS and LDS than the rest of the population (Figure 7E and 7F), and when compared with poor prognosis phenotype (low-MAP17, low-pH2AX and suboptimal cisplatin dose) (Figure 7G and 7H).

DISCUSSION

In this manuscript, we have shown that pH2AX has a prognostic role in patients with laryngeal cancer. We hypothesize, taking into account the combined analysis with p53 and MAP17, that the DDR pathway could have an essential role in laryngeal cancer. Although further research is needed, we think that our results are opening a new window to identify biomarkers that in the future may allow changes in clinical practice, as to date there are no biomarkers that could identify those patients that will benefit from radiotherapy-based treatments instead of surgery.

We found that pH2AX was related to LDS (High-pH2AX HR 0.26, $p = 0.02$) in a cohort of 53 patients with larynx cancer. When analyzed together pH2AX expression and dose of cisplatin received during radical treatment, there is a significant correlation with survival (high-pH2AX and optimal dose of cisplatin 72 months *vs* 38.6 months, $p = 0.03$) and LDS (high-pH2AX and optimal dose of cisplatin 66.9 months *vs* 27 months, $p = 0.019$). Our data suggest that inherent DDR pathway activation (measured by the end-point of phosphorylation of H2AX) is a valuable prognostic marker in patients with laryngeal carcinoma who received preservation approaches. Our data also show the importance of performing optimal

cisplatin treatment for tumor response. However, the fact that unexpected radiotherapy delays and interruptions did not affect survival in our cohort could be explained to dose compensations. Radiobiological-based calculations were performed in those patients in order to achieve an equivalent biological effectiveness by adding some more fractions to the overall treatment.

Tumor cells from clinical specimens show constitutive activation of DNA damage signalling as demonstrated by the presence of gH2AX phosphorylation and other DDR signalling proteins [38-40]. This DDR activation was found to peak at early stage tumors, persisting further among malignant tumors mostly by inactivating p53 gatekeeper [40]. It has been proposed that the DDR-network may serve as an inducible barrier to control the initial steps of tumor development by inducing p53-dependent senescence or apoptosis [38-40]. Further ongoing chronic DDR activation favours the outgrowth of malignant clones with genetic or epigenetic defects in DNA-repair mechanism such as those involved in the DDR pathway [40]. Our samples, from already malignant tumors (stages II-IV), in which only a subset of them

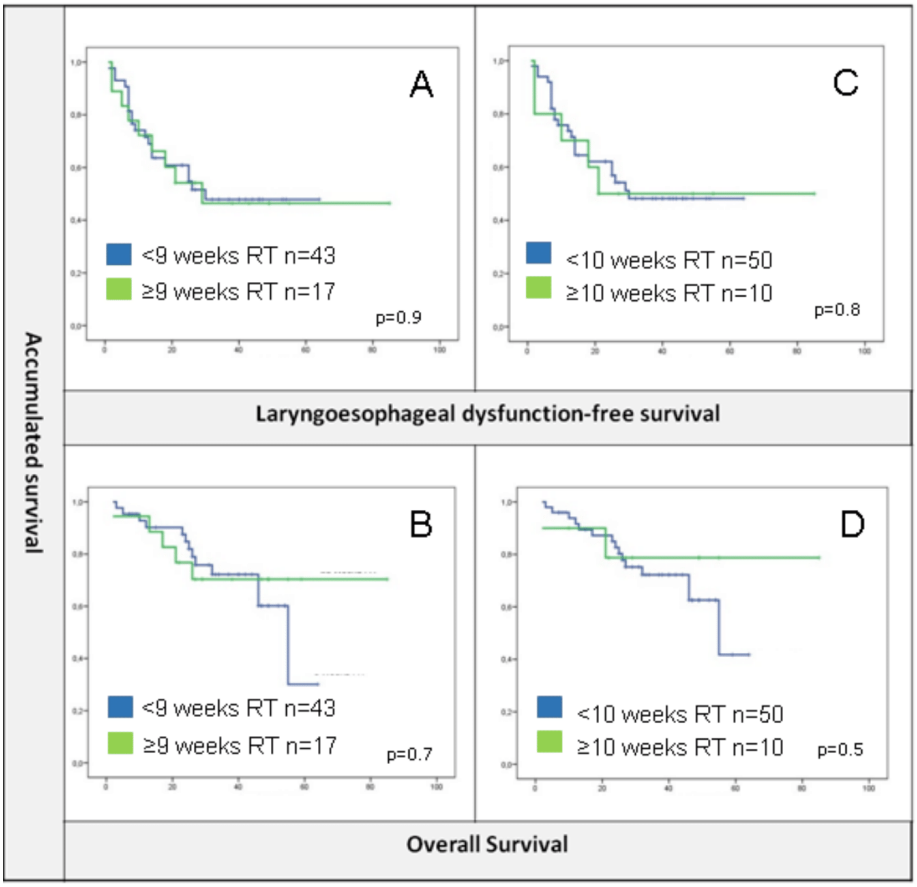

Figure 3: A. and **B.** No differences were found for radiotherapy delivered within less than 9 weeks or ≥9 weeks in terms of LDS or OS. C and D. Same results for a cut-off of 10 weeks.

showed mutant p53, correlated with worse onset of the disease. It is likely DNA-damage defects inducing DDR activation have been carried through the malignant process and it is possible that other proteins are mutated in the process avoiding the requirement for p53 inactivation.

pH2AH is a broad DNA damage marker that appears under different physiological conditions. Senescent cells display molecular characteristics of DNA damage [41-43]. These markers include nuclear foci of phosphorylated histone H2AX, the localization at double-strand break sites of DNA-repair and DNA-damage checkpoint factors, such as 53BP1, MDC1 and NBS1 [44-46]. Senescent cells also contain activated forms of the DNA-damage checkpoint kinases Chk1 and Chk2. During replicative senescence, markers of a DNA damage response localize at telomeres [45, 47], indicating that the DNA damage response is triggered by telomere shortening [48]. Similarly, the redox potential also results in DNA damage and senescence [49]. Very interestingly, oncogene-induced senescence has been found to induce DNA-damage due to an excess of replication forks. This oncogenic-induced hyper-replication signal, or *replication stress*, is associated with persistent DNA-damage [50, 51] inducing senescence [52]. Therefore, not only senescence is viewed as a response to DNA-damage, but DNA-damage as a marker of senescence. In that sense, high pH2AX appeared in early stage tumors and is a marker of good prognosis [38, 53, 54]. However, in our cohort, pH2AX levels are increased in advanced stages of tumors, and contrarily to this hypothesis, are a marker of bad prognosis, indicating that our pH2AX observations are not due to cellular senescence, neither by continuous proliferation nor by replication stress.

In that line, phosphorylation of H2AX is not always a marker of DNA damage. It also can be a marker of activated mTOR, eliciting replicative stress and a

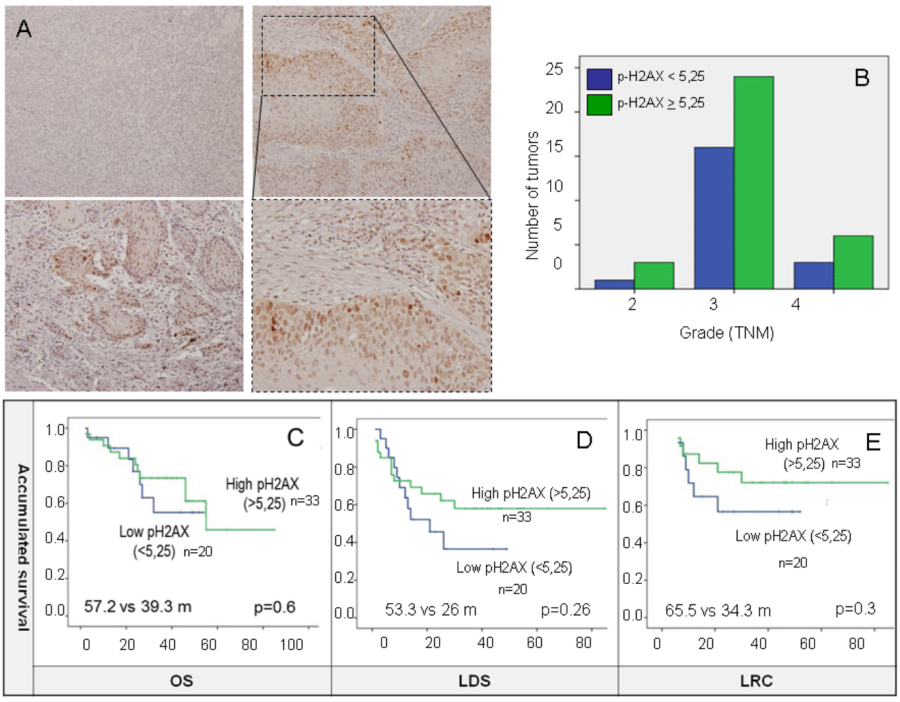

Figure 4: A. Positive pH2AX expression, considered as nuclei staining was shown in 46 (86.8%) samples. **B.** Levels of pH2AX are equally distributed among tumor stages. **C.**, **D.** and **E.** high-pH2AX show a trend towards better OS, LDS and LRC not statistically significant in the Kaplan-Meier analysis. 5.25 as indicated by the ROC curve were used as cut-off for defining high and low expression of pH2AX for survival analysis.

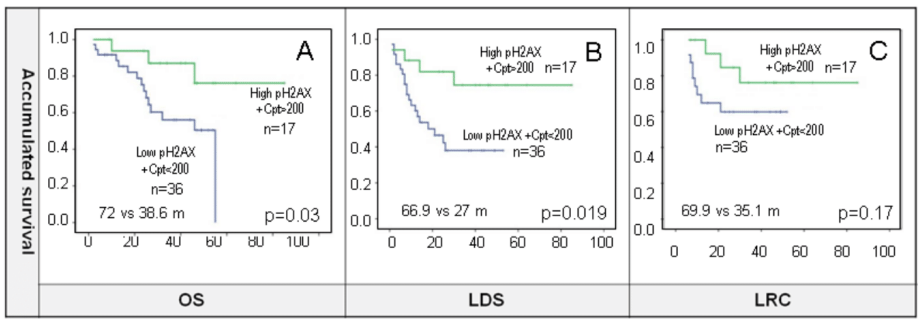

Figure 5: A., B. and **C.** pH2AX and dose of concomitant cisplatin were combined in a new variable where high-pH2AX and cisplatin (Cpt) ≥200 mg/m2 was considered as good prognosis phenotype category. The results show improved OS and LDS in this subgroup, and a trend towards better LRC. 5.25 as indicated by the ROC curve were used as cut-off for defining high and low expression of pH2AX for survival analysis.

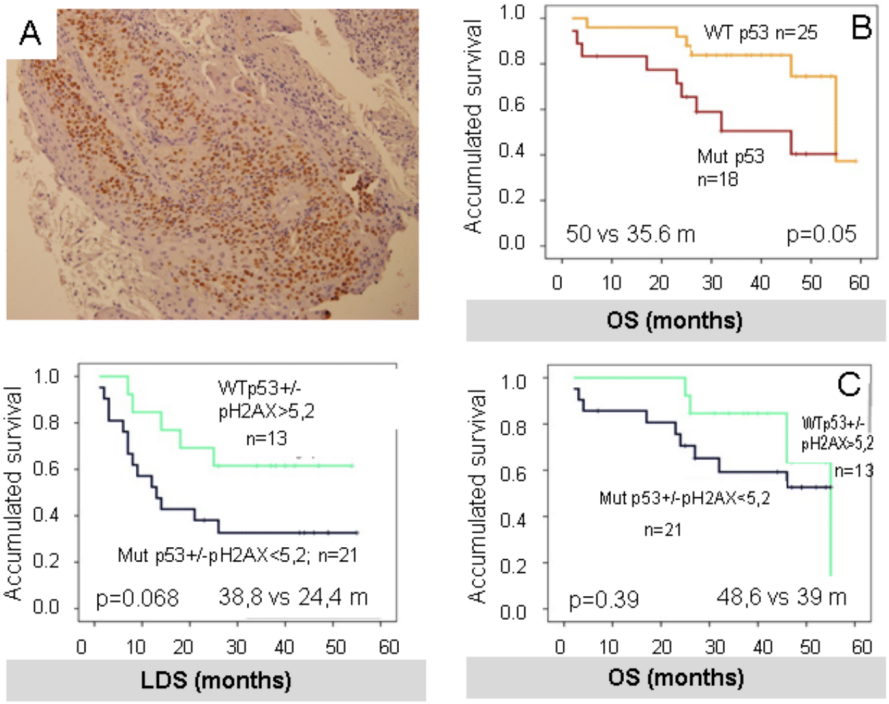

Figure 6: A. P53 was measured as >5% positive nuclei, as shown in the picture. **B.** positive P53 (+ P53) correlates with worse OS in our cohort. **C.** and **D.** results of the combination of P53 and pH2AX in a new variable. Although there was a trend towards better outcomes in the good prognosis phenotype which included negative P53 (-P53) and high-pH2AX, this was not statistically significant for OS and LDS.

pseudo DNA-damage in senescent cells [46, 51, 55-58]. The dynamics of senescence exhibit 2 different steps: cell cycle arrest and further acquisition of senescence features, which includes permanent arrest, termed *geroconversion* [51, 58-60]. If geroconversion is not activated, cells are only transiently arrested with the possibility of resuming growth once the proliferation constraints have been eliminated [44, 61]. It has also been shown that if mTOR is activated under conditions of proliferative arrest, then arrest becomes permanent and the cell undergoes senescence [59, 60, 62]. Under these conditions of cell cycle arrest and mTOR activation, the phosphorylation of H2AX is launched, becoming a marker of cellular senescence [46, 52, 55, 62]. In fact, rapamycin treatment, which inhibits mTOR, can divert senescence into quiescence, allowing the cell to resume growth once conditions are more favorable [63-66]. Since mTOR is the master regulator of protein synthesis [67], it has been proposed that this contribution is due to the function of mTOR as a sensor of cellular nutrients and energy status as well as growth factor signals [68, 69]. However, it has also been reported that mTOR activation in the context of growth arrest is perceived by the cells as and unwanted oncogenic signal, activating the replicative stress and pseudo DNA-damage signaling [46, 52, 55, 62]. In any case, high levels of pH2AX as marker of cellular senescence should be associated to better prognosis, and to some extent to early stage tumors. However, it will be of interest to correlate the levels of pH2AH with those of mTOR activation in laryngeal tumors to provide a more accurate hypothesis of the pH2AH inducers.

Our data show that high levels of pH2AX correlate with better prognosis after treatment with DNA-damage agents such as cisplatin and radiotherapy, especially if cisplatin is given at optimal doses. These data are suggestive of a collaboration of DDR pathway activation, perhaps as an indicator of low DNA-repair ability and DNA-damaging agents in tumor therapy. The fact that doses of cisplatin are important for survival (Figure 2) seems to confirm this hypothesis. In line with this, wt-P53 with high levels of pH2AX conforms a subgroup of good prognosis (Figure 6) suggesting that P53 activity is essential to drive physiological response to apoptosis (or senescence) of DNA-damage agents in tumors with DDR activated.

These data are opposite to the found in early operable non-small cell lung cancer (NSCLC). In this study, low levels of pH2AX correlated with better survival outcomes. The combination of wild type p53 and low-phosphorylated γH2AX phenotype showed also better survival. However, NSCLC patients were treated with surgery and not with radiotherapy [30]. This lack of treatment with radiotherapy could be the cause of the different behavior respect the pH2AX. Radiotherapy increases oxidative stress and reactive oxygen species that in combination with preexisting DNA damage can increase cell damage above threshold inducing increased tumor efficacy. Our data support this hypothesis since combination with another ROS-inducing agent such as cisplatin is essential to gain better survival in these patients. Furthermore, the combination of MAP17, a known ROS-inducing oncogene [32, 35, 36, 70] also supports the essential role of radiotherapy in this response.

We have recently shown that MAP17 levels, a small non-glycosylated membrane protein overexpressed in carcinomas, are associated with overall survival ($p < 0.001$) and laryngoesophageal dysfunction-free survival ($p = 0.002$) [32]. MAP17 increases endogenous ROS [35, 36, 70]. ROS are well known mediators of DNA damage [37]. Our data suggest that high levels of MAP17 induced ROS that in turn increases DNA-damage and DDR signaling. Upon further DNA-damage and further increase in ROS molecules induced by cisplatin and RT treatment, tumors with higher oxidative stress (higher MAP17, higher ROS denoted by higher pH2AX), are more suitable to undergo apoptosis in the presence of P53 activity. Therefore, our data seems to confirm that pH2AX is a marker of structural DNA-damage in the laryngeal tumors that may become a novel and valuable prognostic biomarker for laryngeal carcinoma.

MATERIALS AND METHODS

Patient's characteristics and treatment

We evaluated 63 patients with larynx cancer from August 2005 to February 2014. However, out of the 63 tumoral samples, only 53 of them could be studied. All samples were obtained from diagnostic biopsies before any treatment. All patients completed the informed consent form and the project was approved by the local ethical committee at the HUVR (PI13/059). Patients received treatment in our institution but tumor samples were obtained from four different national hospitals where the diagnosis was made. Eligibility criteria for preservation include patients with stage II-IV laryngeal tumors that had no contraindication for chemotherapy and/or radiotherapy, significant cartilage destruction, or more than 2 cm tumoral invasion of the base of the tongue. TNM Staging System for the Larynx (7th ed., 2010) was used for tumor classification. This cohort has been previously reported in [32].

Tissue acquirement and preparation

Formalin-fixed, paraffin-embedded tissue sections from 63 laryngeal carcinomas were selected with the collaboration of the Andalusian Health Care Biological Resource Centre. Histological characterization of all samples was done by Hematoxylin and Eosin staining,

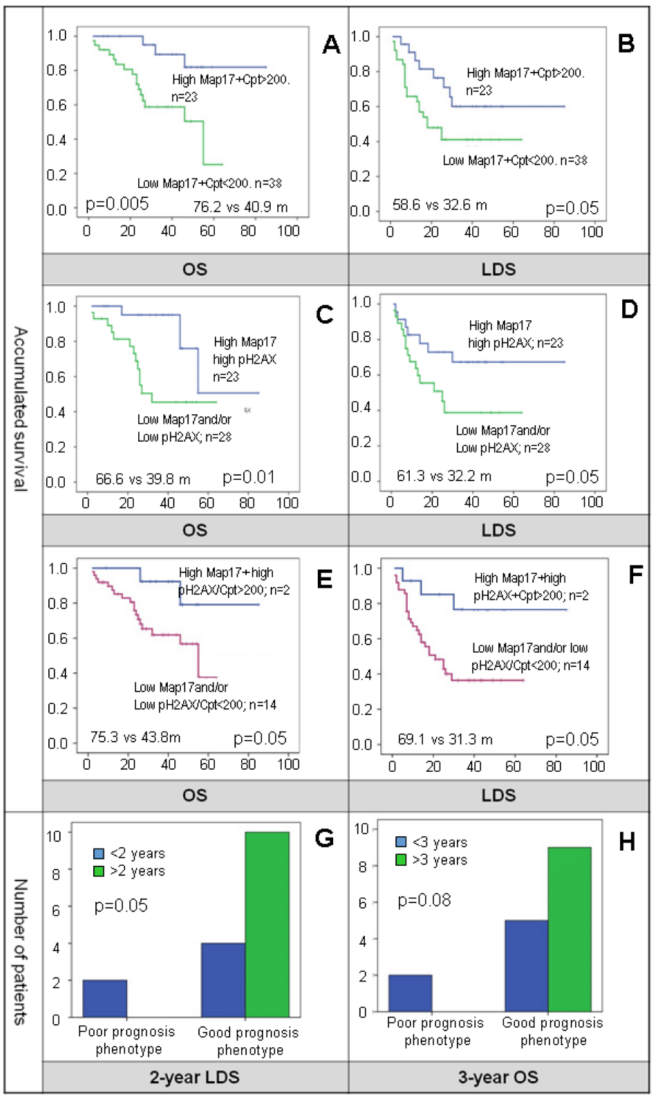

Figure 7: A. and **B.** the combination of high-MAP17 and optimal doses of cisplatin (Cpt) showed better OS and LDS. **C.** and **D.** patients with high-MAP17 and high-pH2AX with higher structural DNA-damage showed to have better OS and LDS. **E.** and **F.** survival for patients with high-pH2AX, high-MAP17 and optimal dose of cisplatin was statistically better. **G.** and **H.** the subgroup of patients with high-pH2AX, high-MAP17 and cisplatin optimal dose patients was compared to the patients that had low-pH2AX, low-MAP17 and did not complete cisplatin. Although limited in numbers, none of the patients with poor prognosis phenotype reached more than 2-years LDS or more than 3-years OS.

followed by immunohistochemistry (IHQ) analysis of tissue microarrays (TMA).

Immunohistochemistry

Three-micrometer slices were sectioned from the TMA block and applied to coated, immunochemistry slides (DAKO, Glostrup, Denmark). The slides were baked overnight in a 56°C oven, deparaffinized in xylene for 20 min, rehydrated through a graded ethanol series and washed with PBS. A heat-induced epitope retrieval step was performed by heating a slide in a solution of sodium citrate buffer pH 6.5 for 2 min in a conventional pressure cooker. After heating, the slides were incubated with proteinase K for 10 min and rinsed in cool running water for 5 min. Endogenous peroxide activity was quenched with 1.5% hydrogen peroxide (DAKO) in methanol for 10 minutes, and incubation with the primary antibodies anti-gamma H2A.X (phospho S139) antibody (ab11174 from Abcam) and anti-p53: p53 FL 393 (sc-6243 from Santa Cruz); was performed for 40 min. After incubation, immunodetection was performed with the EnVision (DAKO, Glostrup, Denmark) visualization system using diaminobenzidinechromogen as the substrate, according to the manufacturer's instructions. Immunostaining was performed in a TechMate 500 automatic immunostaining device (DAKO) and measured through a double-blind visual assessment using microscopic observation according to the anatomopathological experience of pathologists. Sample scoring was performed by microscopic analysis, considering the percentage of nuclei stained cells.

Statistical analysis and definitions

Kaplan-Meier method was used for survival analysis, using Cox Proportional Hazards model to adjust for the explanatory variables, obtain the p-values and estimate the hazard ratios (HR). Multivariate logistic regression was used to obtain odds ratio (OR) and confidence intervals (CI 95%). Pearson's correlation measured dependence between quantitative variables. A receiver operating characteristic (ROC) curve was performed to assess pH2AX cutoff point (two-year OS), which we checked using the optimal Youden index-based point. In addition, a log-rank test compared the survival distributions between high and low PH2AX both as a single variable and in combination with cisplatin optimal dose, MAP17 and p53. Categorical data were studied with contingency tables that included Chi-square statistics. Calculations were performed using SPSS 15.0 software.

OS has been defined as the length of time from diagnosis until the last medical record. LCR was measured as length of time from diagnosis until the relapse or last medical record, in those patients who did not develop distant metastases or died due to different causes than the tumor. For laryngoesophageal dysfunction-free survival (LDS) we adopted Lefebvre Larynx Preservation Consensus Panel that included as endpoint events: death, local relapse, total or partial laryngectomy, tracheotomy at two or more years, or the presence of a feeding tube at two or more years [33].

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

REFERENCES

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010; 127: 2893-2917.
2. de Miguel-Luken MJ, Chaves-Conde M, Carnero A. A Genetic view of Laryngeal Cancer heterogeneity. Cell Cycle. 2016; 15:1202-12.
3. Talamini R, Bosetti C, La Vecchia C, Dal Maso L, Levi F, Bidoli E, Negri E, Pasche C, Vaccarella S, Barzan L, Franceschi S. Combined effect of tobacco and alcohol on laryngeal cancer risk: a case-control study. Cancer causes & control. 2002; 13: 957-964.
4. Shangina O, Brennan P, Szeszenia-Dabrowska N, Mates D, Fabianova E, Fletcher T, t'Mannetje A, Boffetta P, Zaridze D. Occupational exposure and laryngeal and hypopharyngeal cancer risk in central and eastern Europe. Am J Epidemiol. 2006; 164: 367-375.
5. Negri E, Boffetta P, Berthiller J, Castellsague X, Curado MP, Dal Maso L, Daudt AW, Fabianova E, Fernandez L, Wunsch-Filho V, Franceschi S, Hayes RB, Herrero R, Koifman S, Lazarus P, Lence JJ et al. Family history of cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. Int J Cancer. 2009; 124: 394-401.

6. Torrente MC, Rodrigo JP, Haigentz M, Jr., Dikkers FG, Rinaldo A, Takes RP, Olofsson J, Ferlito A. Human papillomavirus infections in laryngeal cancer. Head Neck. 2011; 33: 581-586.
7. Gao X, Fisher SG, Mohideen N, Emami B. Second primary cancers in patients with laryngeal cancer: a population-based study. Int J Radiat Oncol Biol Phys. 2003; 56: 427-435.
8. Sessions DG. Surgical pathology of cancer of the larynx and hypopharynx. Laryngoscope. 1976; 86: 814-839.
9. Singh B, Bhaya M, Stern J, Roland JT, Zimbler M, Rosenfeld RM, Har-El G, Lucente FE. Validation of the Charlson comorbidity index in patients with head and neck cancer: a multi-institutional study. Laryngoscope. 1997; 107: 1469-1475.
10. Mayne ST, Cartmel B, Kirsh V, Goodwin WJ, Jr. Alcohol and tobacco use prediagnosis and postdiagnosis, and survival in a cohort of patients with early stage cancers of the oral cavity, pharynx, and larynx. Cancer Epidemiol Biomarkers Prev. 2009; 18: 3368-3374.
11. Di Martino E, Sellhaus B, Hausmann R, Minkenberg R, Lohmann M, Esthofen MW. Survival in second primary malignancies of patients with head and neck cancer. J Laryngol Otol. 2002; 116: 831-838.
12. Raitiola H, Pukander J, Laippala P. Glottic and supraglottic laryngeal carcinoma: differences in epidemiology, clinical characteristics and prognosis. Acta Otolaryngol. 1999; 119: 847-851.
13. Zhang SY, Lu ZM, Luo XN, Chen LS, Ge PJ, Song XH, Chen SH, Wu YL. Retrospective analysis of prognostic factors in 205 patients with laryngeal squamous cell carcinoma who underwent surgical treatment. PLoS One. 2013; 8: e60157.
14. Yilmaz T, Hosal S, Ozyar E, Akyol F, Gursel B. Post-operative radiotherapy in advanced laryngeal cancer: effect on local and regional recurrence, distant metastases and second primaries. J Laryngol Otol. 2005; 119: 784-790.
15. Herchenhorn D, Dias FL, Ferreira CG, Araujo CM, Lima RA, Small IA, Kligerman J. Impact of previous tracheotomy as a prognostic factor in patients with locally advanced squamous cell carcinoma of the larynx submitted to concomitant chemotherapy and radiation. ORL; journal for oto-rhino-laryngology and its related specialties. 2008; 70: 381-388.
16. Wolf GT, Hong WK. Induction chemotherapy for organ preservation in advanced laryngeal cancer: is there a role? Head Neck. 1995; 17: 279-283.
17. Chevalier D, Laccourreye O, Brasnu D, Laccourreye H, Piquet JJ. Cricohyoidoepiglottopexy for glottic carcinoma with fixation or impaired motion of the true vocal cord: 5-year oncologic results with 112 patients. The Annals of otology, rhinology, and laryngology. 1997; 106: 364-369.
18. Silver CE, Beitler JJ, Shaha AR, Rinaldo A, Ferlito A. Current trends in initial management of laryngeal cancer: the declining use of open surgery. Eur Arch Otorhinolaryngol. 2009; 266: 1333-1352.
19. Jorgensen K, Godballe C, Hansen O, Bastholt L. Cancer of the larynx—treatment results after primary radiotherapy with salvage surgery in a series of 1005 patients. Acta Oncol. 2002; 41: 69-76.
20. American Society of Clinical O, Pfister DG, Laurie SA, Weinstein GS, Mendenhall WM, Adelstein DJ, Ang KK, Clayman GL, Fisher SG, Forastiere AA, Harrison LB, Lefebvre JL, Leupold N, List MA, O'Malley BO, Patel S et al. American Society of Clinical Oncology clinical practice guideline for the use of larynx-preservation strategies in the treatment of laryngeal cancer. J Clin Oncol. 2006; 24: 3693-3704.
21. Forastiere AA, Goepfert H, Maor M, Pajak TF, Weber R, Morrison W, Glisson B, Trotti A, Ridge JA, Chao C, Peters G, Lee DJ, Leaf A, Ensley J, Cooper J. Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. N Engl J Med. 2003; 349: 2091-2098.
22. Forastiere AA, Zhang Q, Weber RS, Maor MH, Goepfert H, Pajak TF, Morrison W, Glisson B, Trotti A, Ridge JA, Thorstad W, Wagner H, Ensley JF, Cooper JS. Long-term results of RTOG 91-11: a comparison of three nonsurgical treatment strategies to preserve the larynx in patients with locally advanced larynx cancer. J Clin Oncol. 2013; 31: 845-852.
23. Pignon JP, le Maitre A, Maillard E, Bourhis J, Group M-NC. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomised trials and 17,346 patients. Radiother Oncol. 2009; 92: 4-14.
24. Ang KK, Zhang Q, Rosenthal DI, Nguyen-Tan PF, Sherman EJ, Weber RS, Galvin JM, Bonner JA, Harris J, El-Naggar AK, Gillison ML, Jordan RC, Konski AA, Thorstad WL, Trotti A, Beitler JJ et al. Randomized phase III trial of concurrent accelerated radiation plus cisplatin with or without cetuximab for stage III to IV head and neck carcinoma: RTOG 0522. J Clin Oncol. 2014; 32: 2940-2950.
25. Ghi MG, Paccagnella A, D'Amanzo P, Mione CA, Fasan S, Paro S, Mastromauro C, Carnuccio R, Turcato G, Gatti C, Pallini A, Nascimben O, Biason R, Oniga F, Medici M, Rossi F et al. Neoadjuvant docetaxel, cisplatin, 5-fluorouracil before concurrent chemoradiotherapy in locally advanced squamous cell carcinoma of the head and neck *versus* concomitant chemoradiotherapy: a phase II feasibility study. Int J Radiat Oncol Biol Phys. 2004; 59: 481-487.
26. Strojan P, Vermorken JB, Beitler JJ, Saba NF, Haigentz M, Jr., Bossi P, Worden FP, Langendijk JA, Eisbruch A, Mendenhall WM, Lee AW, Harrison LB, Bradford CR, Smee R, Silver CE, Rinaldo A et al. Cumulative cisplatin dose in concurrent chemoradiotherapy for head and neck cancer: A systematic review. Head Neck. 2016; 38 Suppl 1:E2151-8.

27. Machtay M, Moughan J, Trotti A, Garden AS, Weber RS, Cooper JS, Forastiere A, Ang KK. Factors associated with severe late toxicity after concurrent chemoradiation for locally advanced head and neck cancer: an RTOG analysis. J Clin Oncol. 2008; 26: 3582-3589.
28. Karp JE, Ricklis RM, Balakrishnan K, Briel J, Greer J, Gore SD, Smith BD, McDevitt MA, Carraway H, Levis MJ, Gandhi V. A phase 1 clinical-laboratory study of clofarabine followed by cyclophosphamide for adults with refractory acute leukemias. Blood. 2007; 110: 1762-1769.
29. Ivashkevich A, Redon CE, Nakamura AJ, Martin RF, Martin OA. Use of the gamma-H2AX assay to monitor DNA damage and repair in translational cancer research. Cancer Lett. 2012; 327: 123-133.
30. Matthaios D, Foukas PG, Kefala M, Hountis P, Trypsianis G, Panayiotides IG, Chatzaki E, Pantelidaki E, Bouros D, Karakitsos P, Kakolyris S. gamma-H2AX expression detected by immunohistochemistry correlates with prognosis in early operable non-small cell lung cancer. OncoTargets and therapy. 2012; 5: 309-314.
31. Brunner AH, Hinterholzer S, Riss P, Heinze G, Weiss K, Brustmann H. Expression of gamma-H2AX in endometrial carcinomas: an immunohistochemical study with p53. Gynecol Oncol. 2011; 121: 206-211.
32. de Miguel-Luken MJ, Chaves-Conde M, de Miguel-Luken V, Munoz-Galvan S, Lopez-Guerra JL, Mateos JC, Pachon J, Chinchon D, Suarez V, Carnero A. MAP17 (PDZKIP1) as a novel prognostic biomarker for laryngeal cancer. Oncotarget. 2015; 6: 12625-12636. doi:10.18632/oncotarget.3470.
33. Lefebvre JL, Ang KK, Larynx Preservation Consensus P. Larynx preservation clinical trial design: key issues and recommendations-a consensus panel summary. Int J Radiat Oncol Biol Phys. 2009; 73: 1293-1303.
34. Ashraf MJ, Maghbul M, Azarpira N, Khademi B. Expression of Ki67 and P53 in primary squamous cell carcinoma of the larynx. Indian journal of pathology & microbiology. 2010; 53: 661-665.
35. Guijarro MV, Leal JF, Blanco-Aparicio C, Alonso S, Fominaya J, Lleonart M, Castellvi J, Ramon y Cajal S, Carnero A. MAP17 enhances the malignant behavior of tumor cells through ROS increase. Carcinogenesis. 2007; 28: 2096-2104.
36. Carnero A. MAP17 and the double-edged sword of ROS. Biochim Biophys Acta. 2012; 1826: 44-52.
37. Caputo F, Vegliante R, Ghibelli L. Redox modulation of the DNA damage response. Biochem Pharmacol. 2012; 84: 1292-1306.
38. Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, Orntoft T, Lukas J, Bartek J. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature. 2005; 434: 864-870.
39. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio RA, Jr., Kastrinakis NG, Levy B, Kletsas D, Yoneta A, Herlyn M, Kittas C, Halazonetis TD. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature. 2005; 434: 907-913.
40. Bartek J, Bartkova J, Lukas J. DNA damage signalling guards against activated oncogenes and tumour progression. Oncogene. 2007; 26: 7773-7779.
41. Blagosklonny MV. Cell cycle arrest is not yet senescence, which is not just cell cycle arrest: terminology for TOR-driven aging. Aging (Albany NY). 2012; 4: 159-165. doi: 10.18632/aging.100443.
42. Blagosklonny MV. Hypoxia, MTOR and autophagy: converging on senescence or quiescence. Autophagy. 2013; 9: 260-262.
43. Carnero A. Markers of cellular senescence. Methods Mol Biol. 2013; 965: 63-81.
44. Ruiz L, Traskine M, Ferrer I, Castro E, Leal JF, Kaufman M, Carnero A. Characterization of the p53 response to oncogene-induced senescence. PLoS ONE. 2008; 3: e3230.
45. d'Adda di Fagagna F. Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer. 2008; 8: 512-522.
46. Pospelova TV, Demidenko ZN, Bukreeva EI, Pospelov VA, Gudkov AV, Blagosklonny MV. Pseudo-DNA damage response in senescent cells. Cell Cycle. 2009; 8: 4112-4118.
47. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T, Saretzki G, Carter NP, Jackson SP. A DNA damage checkpoint response in telomere-initiated senescence. Nature. 2003; 426: 194-198.
48. Blasco MA. Telomere length, stem cells and aging. Nat Chem Biol. 2007; 3: 640-649.
49. Blagosklonny MV. Aging: ROS or TOR. Cell Cycle. 2008; 7: 3344-3354.
50. Fumagalli M, Rossiello F, Mondello C, d'Adda di Fagagna F. Stable cellular senescence is associated with persistent DDR activation. PLoS One. 2014; 9: e110969.
51. Leontieva OV, Lenzo F, Demidenko ZN, Blagosklonny MV. Hyper-mitogenic drive coexists with mitotic incompetence in senescent cells. Cell Cycle. 2012; 11: 4642-4649.
52. Darzynkiewicz Z. When senescence masquerades as DNA damage: is DNA replication stress the culprit? Cell Cycle. 2009; 8: 3810-3811.
53. Nuciforo PG, Luise C, Capra M, Pelosi G, d'Adda di Fagagna F. Complex engagement of DNA damage response pathways in human cancer and in lung tumor progression. Carcinogenesis. 2007; 28: 2082-2088.
54. Bartkova J, Hamerlik P, Stockhausen MT, Ehrmann J, Hlobilkova A, Laursen H, Kalita O, Kolar Z, Poulsen HS, Broholm H, Lukas J, Bartek J. Replication stress and oxidative damage contribute to aberrant constitutive activation of DNA damage signalling in human gliomas. Oncogene. 2010; 29: 5095-5102.


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